

Temperature effects on the development in the solitary bee *Osmia bicornis* (Hymenoptera, Megachilidae)

Development time, brain microstructure, and
cognitive abilities



Dissertation zur Erlangung
des Doktorgrades der Naturwissenschaften (Dr. rer. nat.) der Fakultät für
Biologie und vorklinische Medizin der Universität Regensburg

vorgelegt von
Sabine Radmacher

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im Jahr 2011

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Unterschrift:

„Oh glücklich! wer noch hoffen kann
aus diesem Meer des Irrtums aufzutauchen.
Was man nicht weiß das eben brauchte man,
und was man weiß kann man nicht brauchen.“

Johann Wolfgang von Goethe, „Faust - Der Tragödie erster Teil“

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LIST OF PUBLICATIONS

This thesis is based on the following manuscripts:

- Radmacher, S. and Strohm, E. (2011)** Effects of constant and fluctuating temperatures on the development of the solitary bee *Osmia bicornis* (Hymenoptera; Megachilidae). *Apidologie*, accepted. (chapter 2)
- Radmacher, S. and Strohm, E.** Temperature effects on solitary bee development - does temperature experienced as larva influence the duration of the prepupal stage? *Apidologie*, submitted. (chapter 3)
- Radmacher, S. and Strohm, E.** Effects of temperature during development on the synaptic organization in the brain of the solitary bee *Osmia bicornis* (Hymenoptera, Megachilidae). *Arthropod Structure & Development*, submitted. (chapter 4)
- Radmacher, S. and Strohm, E.** Does temperature experienced during metamorphosis affect the synaptic organization in the brain of a solitary bee? (in preparation). (chapter 5)
- Radmacher, S. and Strohm, E.** Does developmental temperature influence the learning abilities in the solitary bee *Osmia bicornis* (Hymenoptera, Megachilidae)? (in preparation). (chapter 6)

CHAPTER 1

GENERAL INTRODUCTION

1.1 Temperature – an important environmental factor for insects

Temperature is ecologically the most important physical factor among the multitude of physical and biotic factors that describe the niche of an animal (Cossins and Bowler 1987). Due to its direct influence on many biochemical processes (Johnston and Wilson 2006), temperature has pervasive effects on complex biological processes like development, growth, reproduction, and ageing (Cossins and Bowler 1987). Thus, “virtually everything that an organism does is influenced by and dependent on its thermal condition” (Johnston and Bennett 1996, p. xii (preface)). Ambient temperature is highly variable on several temporal and spatial scales, particularly in temperate regions, and animals had to evolve adaptations to cope with changing and sometimes unpredictable as well as extreme temperatures. Endothermic organisms evolved the capability for active regulation of body temperature that enables them to keep their body temperature in a suitable, relatively narrow range, largely independent of ambient temperatures. Ectothermic organisms, by contrast, have limited ability to regulate their body temperature and, thus, lack in internal thermal homeostasis (Begon et al. 1996; Bale and Hayward 2010). However, though ectotherms are usually largely poikilothermic, they are not simply “at the mercy of environmental temperatures” since they “deploy a range of subtle molecular and organismic strategies in adapting to the vagaries of a thermally unstable environment” (Wieser 1973a, p. 1; 1973b, p. V (preface)). Insects are essentially ectothermic organisms and their physiological processes display a high degree of sensitivity to ambient temperatures (Beck 1983). However, since insects experience extreme temperature variations in time and space, they “have evolved some of the most amazing feats of thermal adaptation and thermoregulation in the entire animal kingdom” (Heinrich 1993, p. 1).

In the course of the current climate change, ambient temperatures are changing. The overall mean temperatures are rising, spring (i.e. flowering) starts earlier, the growing season is

lengthened, the intensity and frequency of weather extremes such as heat waves in summer are increasing – and these trends are predicted to continue (Menzel et al. 2006; Schwartz et al. 2006; IPCC 2007; EEA 2008). As a consequence, shifts in phenology and distribution of plants and animals, the disruption of species interactions and the extinction of some species are predicted and have been already reported (Clarke 1996; Harrison 2000; Hughes 2000; Inouye et al. 2000; Fitter and Fitter 2002; Root et al. 2003; Kudo et al. 2004; Winder and Schindler 2004; Jump and Peñuelas 2005; Visser and Both 2005; Parmesan 2006; Estrella et al. 2007; Deutsch et al. 2008; Amano et al. 2010; Yang and Rudolf 2010). Due to their sensitivity to ambient temperatures, their short generation time as well as their diversity and abundance, insects are preferred organisms for the investigation of influences of climate change on biological systems (Bale et al. 2002; Menéndez 2007; Deutsch et al. 2008; Bale and Hayward 2010; Robinet and Roques 2010). While herbivorous insects have been studied with regard to potential consequences of climate change on several life history traits like development time, reproduction, and voltinism (e.g. Bale et al. 2002; Altermatt 2010; Musolin et al. 2010) due to their role as pests, the direct impact on such life history traits of the beneficial pollinators received less attention. Only phenological shifts in the appearance of pollinators and a resulting disruption in plant-pollinator interactions have been examined (Kudo et al. 2004; Gordo and Sanz 2005; Memmott et al. 2007; Hegland et al. 2009).

Insects, particularly bees, are generally the most important pollinators for crops and many natural plant populations (McGregor 1976; Free 1993; James and Pitts-Singer 2008). Besides the well-known social honeybee, the so-called “wild bees”, including many solitary species, play an important role for pollination (Batra 1995; Freitas and Perreira 2004; Winfree et al. 2007; Kremen 2008), but they are already declining due to habitat loss and fragmentation, agricultural intensification, and pesticide use (Kearns et al. 1998; Steffan-Dewenter et al. 2005; Biesmeijer et al. 2006; Potts et al. 2010). The impact of climate change on pollinators could aggravate this pollinator crisis and cause serious ecologic as well as economic consequences. However, for an estimation of potential consequences of climate change on bees, fundamental knowledge about their thermal requirements and restrictions would be required.

Some solitary bee species belonging to the Megachilidae, particularly the leaf-cutter bee *Megachile rotundata* and some species of the genus *Osmia*, received increasing interest during the last decades since they are effective and manageable pollinators for alfalfa and

fruit crops. *M. rotundata* has already been managed for decades in a large scale for alfalfa pollination in the USA and Canada and some mason bees (*Osmia* spp.) are on the brink of commercial scale-use (Free 1993; Krunic and Stanisavljević 2006a; James and Pitts-Singer 2008). In order to support the development of suitable methods for management and winter storage, temperature influences on development, overwintering, survival, and emergence in some *Megachile* and *Osmia* species have been investigated (Tasei and Masure 1978; Tepedino and Parker 1986; Richards et al. 1987; Olafir 1989; Whitfield and Richards 1992; Bosch and Blas 1994; Rust 1995; Wilson and Abel 1996; Bosch and Kemp 2000, 2003, 2004; Bosch et al. 2000, Kemp and Bosch 2000, 2001, 2005; Krunic and Stanisavljević 2006b; Yocum et al. 2006; Sheffield et al. 2008; White et al. 2009; Sgolastra et al. 2010). However, as in most studies about temperature effects on insects (Beck 1983; Cossins and Bowler 1987), almost only constant temperatures or maximal one fluctuating temperature regime were used in these studies on solitary bees. For commercial management purposes, the results of these studies might be meaningful and important, but in view of the fact that constant temperatures are rarely experienced in nature, such work is of limited ecological significance (Cossins and Bowler 1987) since constant and fluctuating temperature regimes might differ in their effect on several life history traits in insects - as it has been already shown for development time (Beck 1983; Ratte 1984).

1.2 Temperature and bee development

The temperature experienced during development strongly affects morphology and many important life history traits in ectotherms (Steigenga and Fischer 2009), e.g. development time, growth, body size, and fecundity (Ratte 1984; Cossins and Bowler 1987; Atkinson 1994; Stillwell and Fox 2005). Social insects like termites, ants, social wasps and bees have evolved a variety of passive and active mechanisms that enable them to regulate the microclimate in their nests and, thus, for their brood (reviewed in Seeley and Heinrich 1981; Jones and Oldroyd 2006). Besides passive thermoregulation through nest site selection, nest orientation and architecture (Seeley and Morse 1978; Jaycox and Parise 1981; Seeley 1982), the well-known social honeybees use active mechanisms like clustering and generating metabolic heat for direct incubation (Free and Simpson 1963; Kronenberg and Heller 1982; Fahrenholz et al. 1989; Bujok et al. 2002; Kleinhenz 2003; Stabentheiner et al. 2003, 2010) as well as fanning and water evaporation (Hess 1926; Lindauer 1954;

Seeley and Heinrich 1981; Southwick and Moritz 1987; Oldroyd and Wongsiri 2006) for the regulation of in-nest temperatures. While ambient temperatures as well as temperatures in the hive outside the brood area may show remarkable fluctuations, the temperature in the brood area is maintained in a narrow range of 33-36°C, mostly around 34-35°C (Hess 1926; Himmer 1932; Kronenberg and Heller 1982; Jones et al. 2004). Adult honeybees tolerate a considerable range of temperature. However, survival and regular growth of the brood apparently depends upon brood nest temperatures being maintained in the above mentioned narrow range (Kronenberg and Heller 1982; Mardan and Kevan 2002) since warmer and cooler temperatures had clear negative effects like elevated mortality and malformations (Himmer 1927; Mardan and Kevan 2002). Moreover, even small deviations from the optimal temperature range of 34-35°C affected mortality and susceptibility to pesticides (Medrzycki et al. 2010) and less obvious traits like behavioural performance (Tautz et al. 2003; Becher et al. 2009), short-term learning and memory (Tautz et al. 2003; Jones et al. 2005), as well as the synaptic organization in the brain of adult honeybees (Groh et al. 2004, 2006). Thus, honeybees seem to have adapted to more or less constant temperatures of about 34-35°C during development. Since the temperature maintenance in the brood nest takes much time and energetic effort – Tautz et al. (2003) estimated that about 40% of the energy brought back into the nest in one year (in the form of sugar contained in nectar) is used for heating of the brood nest - honeybees can provide such constant temperature conditions for their brood probably only due to their social way of life that includes overlapping generations and task partitioning.

Unlike the eusocial honeybees, most bee species are solitary (Michener 2000). In solitary bees, every female has to cope with the different tasks in a bees' life - e.g. nest building, nest defence, reproduction, and foraging - on its own. Thus, solitary bees cannot afford the time and energy that would be required for active regulation of brood temperature. They may use some of the passive mechanisms like the selection of a naturally insulated nesting site deep in the substrate, e.g. soil or wood. However, since most solitary bees apparently take no further actions in respect of temperature management for their brood, particularly the offspring of cavity-nesting species that nests above ground (e.g. in plant stems) are largely exposed to the fluctuating ambient temperatures that are probably only slightly buffered by nesting material. If solitary bees showed similar negative effects of deviations from the optimal developmental temperature as honeybees, they would probably suffer huge fitness losses due to fluctuating weather conditions. Thus, solitary bees should have adapted to fluctuating and

changing temperatures during development. For example, they might have evolved broader reaction norms or mechanisms that enable them to compensate for temperature changes during development.

However, where are the limits of these adaptations? Could the current climate change with increasing temperatures negatively influence the development of solitary bees despite their adaptations to withstand temperature fluctuations? At present, our knowledge about potential adaptations to fluctuating and often unpredictable temperatures and the temperature requirements of solitary bees under fluctuating conditions is rather insufficient so that we can only speculate about answers to these questions. Typically, development time of insects decreases with increasing temperature up to the so-called optimal temperature for development which lies usually close to the upper thermal limit (Laudien 1973; Ratte 1984; Cossins and Bowler 1987). However, an accelerated development is not automatically advantageous for each insect species since it could disrupt the synchronisation of the life cycle with the local seasonal environment which is particularly important in temperate regions where insects have to pass adverse winter conditions in a suitable stage (Gotthard 2001; Bale et al. 2002; Bale and Hayward 2010). Moreover, body size usually decreases with increasing temperatures during development according to the so-called “temperature-size rule” (Atkinson 1994, 1996). Body size is related to fitness in many insects (Honěk 1993) and in solitary bees, advantages for larger individuals were shown regarding foraging efficiency, fecundity, nest usurpation, and overwintering survival (Torchio and Tepedino 1980; Tepedino and Torchio 1982, 1994; Sugiura and Maeta 1989; Kim 1997; Bosch and Kemp 2004; Bosch and Vicens 2006; Seidelmann et al. 2010). In addition, if the brain and cognitive abilities were influenced by temperature as it was shown for honeybees (Tautz et al. 2003; Groh et al. 2004, 2006; Jones et al. 2005), the foraging performance of solitary bees could be affected since learning and memory are of crucial importance for foraging performance (Pyke et al. 1977; Hughes et al. 1992). This should be particularly evident in animals like bees that frequently travel between diverse flower patches and their nest (“central place foragers”; Menzel and Müller 1996; Menzel 2001; Giurfa 2007; Dukas 2008; Raine and Chittka 2008) – that applies to social as well as solitary bee species. For solitary bees, decreasing development times as well as decreasing body size with increasing temperature have already been reported (Tasei and Masure 1978; Whitfield and Richards 1992; Bosch and Kemp 2000; Kemp and Bosch 2000; Radmacher and Strohm 2010). However, the respective studies were conducted with (almost) only constant temperature treatments though solitary

bees should be adapted to fluctuating temperature conditions. To our knowledge, potential temperature influences during development on the synaptic organization in the brain or on cognitive abilities of solitary bees have not been investigated so far.

1.3 The study species: *Osmia bicornis*

In our studies, we investigated potential temperature influences during development on several traits (see below) of the solitary Red mason bee, *Osmia bicornis* (Linnaeus 1758; Hymenoptera, Megachilidae). *O. bicornis*, also known as *O. rufa*, is one of the most abundant solitary bees in Central Europe (Raw 1972; Peters 1977; Westrich 1989; Krunic and Stanisavljević 2006a). Three subspecies can be distinguished: The ssp. *rufa* is found in South Sweden, Denmark, and England as well as on the Iberian Peninsula, Sardinia, and Corsica, the ssp. *cornigera* (Rossi 1970) is distributed in Central Europe and wide areas of South and East Europe, and the ssp. *fratricornis* (Pérez 1895) is found on Mallorca and in Northern Africa (Peters 1977; Westrich 1989; Krunic and Stanisavljević 2006a). Our studies were conducted in Regensburg (Germany) where ssp. *cornigera* is native.

O. bicornis displays sexual dimorphism: Females are larger than males and possess two “horns” on their black clypeus as well as an orange “brush” (called scopa) on the ventral side of their abdomen for the transport of pollen. Males have white hairs on the forehead and relatively long antennae (Fig. 1.1). The activity period of this univoltine and polylectic species starts with the emergence of adults in spring (depending on geographical position and weather conditions, but at the earliest at the end of March) and ends with the death of the females at the latest at the beginning of July (Brechtel 1986; Jacobs and Renner 1998). In the studied population, the activity period of about 6-8 weeks usually ranges from early April to mid-June. After mating, females build their preferentially linear nests in a wide range of pre-existing cavities like hollow plant stems, abandoned beetle burrows or holes in walls and roof tiles. They readily accept several kinds of trap nests (Westrich 1989; Müller et al. 1997; Krunic and Stanisavljević 2006a). Each nest consists of several brood cells that are provisioned with pollen from a diverse spectrum of plants and some nectar (< 4 % of total provisions) and separated by loam partitions (Raw 1972; Maddocks and Paulus 1987; Strohm et al. 2002). Females attach a single egg onto the provisions of each brood cell. The larvae hatch, consume their provisions, spin a cocoon, go through a prepupal and a pupal

stage during summer and finally eclose as adults inside their cocoons. The development from oviposition in spring to adult eclosion in autumn usually takes about 14-15 weeks under natural conditions (Raw 1972; Tasei 1973b). The fully developed adults experience a pre-winter phase in autumn until the temperature drops and bees enter the wintering phase. They winter inside their cocoons and emerge the next spring.

Due to its abundance, the acceptance of trap nests, and its capacity as effective and easily manageable pollinator of fruit trees and some other crops, *O. bicornis* has frequently been used for research on solitary bees and their potential as large-scale managed crop pollinators (e.g. Holm 1973; Tasei 1973a, b; Menzel et al. 1988; Seidelmann 1995; van der Steen 1997; Strohm et al. 2002; Steffan-Dewenter 2003; Wilkaniec et al. 2004; Krunić and Stanisavljević 2006a; Neumann and Seidelmann 2006; Konrad et al. 2008; Teper and Biliński 2009; Kornmilch 2010; Radmacher and Strohm 2010; Seidelmann et al. 2010).



Figure 1.1: *O. bicornis* during mating (on the left: frontal; on the right: lateral). The males (with white hair on the forehead) sit on the back of the females.

1.4 Outline of this thesis

In this thesis, we investigated potential influences of temperature during development on development time (chapter 2 and 3), body size (mainly chapter 2 and 3, but also chapter 4 and 5), synaptic organization in the brain (chapter 4 and 5), and the cognitive abilities (chapter 6) of the solitary bee *O. bicornis*. Since the responses of insects to developmental temperature might differ between constant and fluctuating temperature treatments (e.g. Beck

1983; Ratte 1984; Cossins and Bowler 1987) and since the offspring of solitary bees should be adapted to fluctuating rather than constant temperature conditions during development, we used three fluctuating as well as three constant temperature treatments (plus one control group as a reference for natural temperature conditions).

The effect of temperature on development time has been shown to differ among developmental stages of a species (Bosch and Kemp 2000; Rombough 2003; Kemp and Bosch 2005; Folguera et al. 2010) and this might be true for other traits, too. Moreover, the temperature experienced during early (i.e. larval) development might influence the thermal responses of subsequent developmental stages since the insect offspring might somehow acclimatize to the prevailing temperature conditions during (early) development (e.g. Steigenga and Fischer 2009). Therefore, we varied the duration of the exposure to the different temperature treatments in different years. In 2008, we exposed *O. bicornis* offspring to the experimental temperatures during their entire development and investigated the effects on development time, mortality (chapter 2) and the synaptic organization in the mushroom bodies in brains of adult bees (chapter 4). In the following year (2009), we applied the respective temperature treatments only during the development inside the cocoon (i.e. the prepupal and the pupal stage) and likewise investigated the effects on the duration of distinct developmental stages (chapter 3) and the synaptic organization in the brain (chapter 5). The comparison of results of both years (2008 and 2009) might reveal indications for potentially adaptive mechanisms that might help these bees to cope with changing temperatures during development or for any acclimatization to the prevailing temperatures during development. In spring 2010 (after winter diapause), we investigated the learning and memory abilities of bees that developed in 2009, i.e. that experienced the experimental temperatures only during the development inside the cocoon (chapter 6).

Regarding temperature influences on development time and body size, we expected that warmer and fluctuating (vs. constant) temperatures would accelerate the development of *O. bicornis* and that the body size of the bees would decrease with increasing rearing temperatures according to often observed patterns of temperature effects on insect development (e.g. Beck 1983; Ratte 1984; Hagstrum and Milliken 1991; Atkinson 1994). Potential consequences of such effects for *O. bicornis* are discussed in chapter 2 and 3.

The main objective of this thesis was the investigation of temperature influences on the

synaptic organization in the brain and the cognitive abilities in *O. bicornis*, since, to our knowledge, such effects have not yet been explored in a solitary bee. According to studies on honeybees (Groh et al. 2004, 2006), we investigated temperature influences on number and density of distinct synaptic complexes in the calyces of the mushroom bodies (MBs) in the brain of adult *O. bicornis*. The MBs are prominent neuropils in the insect brain that represent multisensory integration centres that play an important role for learning, memory, and orientation (Menzel et al. 1994; Heisenberg et al. 1998; Strausfeld et al. 1998, 2009; Zars 2000; Gronenberg 2001; Fahrbach 2006). We adapted a protocol that was used for honeybees (Groh et al. 2004, 2006) and compared our results for temperature effects on the brain of *O. bicornis* with the respective effects that were found in honeybees (Groh et al. 2004, 2006) to test the hypothesis that solitary bees are adapted and, thus, less susceptible to fluctuating and changing temperatures during development than honeybees (chapter 4 and 5). Thus, we expected to find no or only small effects of developmental temperature on the synaptic organization in the MBs in *O. bicornis*. However, there might be inconspicuous effects of temperature on the brain that do not manifest as observable structural changes but that might nevertheless influence the cognitive abilities of bees – as it was shown for honeybees (Tautz et al. 2003; Jones et al. 2005). Therefore, we developed a visual learning paradigm for megachilid bees and used it for the investigation of potential effects of developmental temperature on learning and fast reversal learning abilities in *O. bicornis* (chapter 6).

CHAPTER 2

EFFECTS OF CONSTANT AND FLUCTUATING TEMPERATURES ON THE DEVELOPMENT OF THE SOLITARY BEE *OSMIA* *BICORNIS* (HYMENOPTERA, MEGACHILIDAE)

Apidologie, accepted

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2.1 SUMMARY

Since the temperature during development may affect growth and fitness in insects, climate change might affect important life history traits of solitary bees. We investigated the impact of three fluctuating and three constant temperature regimes on prepupal weight, mortality and development time of *Osmia bicornis*. Prepupal weight decreased with increasing temperature, but not as strong under fluctuating conditions. Adult mortality increased in the warm treatments. Fluctuating (versus constant) temperatures accelerated development in the most stages and temperature regimes. The duration of almost all developmental phases decreased with increasing temperature, except for the prepupal phase that was prolonged in the warm treatments. The differences in thermal responses to fluctuating vs. constant temperatures illustrated the importance of fluctuating temperatures in studies investigating potential consequences of climate change for insects, including pollinators.

2.2 INTRODUCTION

Temperature is one of the most important environmental factors for ectotherms and the temperature experienced during development strongly affects growth and many life history traits (Stillwell and Fox 2005; Steigenga and Fischer 2009), e.g. body size and development time (Ratte 1984; Atkinson 1994). In contrast to the well-known social honeybees that maintain a more or less constant temperature for their brood (e.g. Jones et al. 2004), most bee species are solitary (Michener 2000) and their offspring are largely exposed to the fluctuating ambient temperatures, at least in cavity-nesting species.

In the course of the current climate change, overall mean temperatures are rising, spring (i.e. flowering) starts earlier, the growing season is prolonged, the intensity and frequency of weather extremes such as heat waves in summer are increasing – and these trends are predicted to progress (Menzel et al. 2006; Schwartz et al. 2006; IPCC 2007; EEA 2008). While some studies explored the potential consequences of climate change for herbivorous insects (e.g. Bale et al. 2002; Musolin et al. 2010) its direct impact on pollinators received little attention. The so-called “wild bees”, including many solitary species, play an important role for the pollination of crops and natural plant populations (Freitas and Pereira 2004; Kremen 2008) and they are already declining due to habitat loss and fragmentation (e.g. Kearns et al. 1998). The impact of climate change on pollinators could aggravate this “pollinator crisis” and cause serious ecologic as well as economic consequences.

In this study, we investigated temperature influences on the development of the European fruit-tree pollinator *Osmia bicornis* (Linnaeus 1758, Hymenoptera, Megachilidae, formerly *O. rufa*). The flight period of this abundant solitary and univoltine species usually ranges from early April to mid-June. Females build their linear nests in pre-existing cavities, provision the brood cells with pollen and some nectar and lay one egg per brood cell. The larvae hatch, consume the provision, spin a cocoon, go through a prepupal and pupal stage and finally eclose as adults in autumn. The development from oviposition to adult eclosion usually takes about 14-15 weeks (Raw 1972; Tasei 1973). The fully developed adults winter inside their cocoons and emerge the next spring.

In ectotherms, higher temperatures usually result in a shorter development time and smaller body size (Ratte 1984; Atkinson 1994). In the univoltine *Osmia* species, the accelerating

effect of high temperatures on development combined with a prolonged growing season (due to climate change) could prolong the pre-wintering period (i.e. the period from adult eclosion inside the cocoon to the onset of wintering temperatures). A long pre-wintering period and small body size, however, had negative effects on survival and fitness in these bees (Tepedino and Torchio 1982; Bosch et al. 2000, 2010; Bosch and Kemp 2004, Bosch and Vicens 2006). Since the prepupal stage seems less susceptible to the negative effects of high temperatures than the adult stage, individuals developing under warm conditions might change the developmental pattern and prolong the prepupal stage (Bosch et al. 2000, 2010; Kemp and Bosch 2005).

Insects are usually adapted to fluctuating natural temperature conditions (see e.g. Beardmore 1960). However, most studies (including these about *Osmia* bees) that investigated temperature influences on development used constant temperatures, with no or only one fluctuating treatment for comparison (e.g. Bosch and Kemp 2000; Kemp and Bosch 2005; Radmacher and Strohm 2010). The effects of fluctuating temperature regimes and the respective constant mean temperatures on development time have often been shown to differ, in many cases with an accelerating effect of fluctuating temperatures (Beck 1983; Ratte 1984). Therefore, the results of laboratory studies using constant temperatures can hardly be extrapolated to natural populations (Beck 1983). Thus, in order to estimate potential consequences of climate change for the respective species, the effects of fluctuating temperatures have to be taken into account.

The main objective of our study was to investigate the effect of both three fluctuating and the respective constant temperatures (cool, medium, warm) on body size, mortality, and the duration of development in *O. bicornis*. With regard to the ongoing climate change, we were particularly interested in whether the bees are affected by warmer temperatures or whether they are able to compensate possible negative effects.

2.3 MATERIALS AND METHODS

2.3.1 Bees

Brood cells were obtained from a free-flying *O. bicornis* population nesting in trap nests in an observation hut near the botanical garden of the University of Regensburg. The trap nests

were made of dense styrofoam covered with transparent polycarbonate lids that allowed continuous observation of the nesting activity and brood development (for more details see Strohm et al. 2002). These trap nests also allowed the separation of recently completed brood cells while the bee female continued nesting in the remaining part of this trap nest. During the peak nesting season (2 to 15 May) in 2008, we used 528 brood cells with 1 to 3 days old eggs from 62 nests for our investigations.

2.3.2 Provision weight

After separation from the nests, the brood cells were brought into the laboratory. The pollen provision with the attached egg was removed with a custom-made scoop to weigh it to the nearest 0.1 mg (Sartorius analytic A120 S). Then the pollen loaf with the egg was carefully put back into its brood cell that was re-sealed with paper on the side and transparent foil on the top, both fixed with adhesive tape.

2.3.3 Temperature treatments

We used six temperature treatments as well as a control group (“Hut”) to investigate possible temperature effects on the bee development. In the three fluctuating regimes temperature followed a sine curve and reached the eponymous minimum and maximum values once in 24 h. The cool, medium, and warm treatments were 10-25°C, 15-30°C, and 20-35°C. The three constant temperatures represented the mean temperatures of the fluctuating regimes: 17.5°C, 22.5°C, and 27.5°C.

The brood cells were randomly allocated to these seven temperature groups immediately after determination of provision weight. The sample sizes were: $N_{\text{Hut}}=66$, $N_{17.5^{\circ}\text{C}}=77$, $N_{22.5^{\circ}\text{C}}=77$, $N_{27.5^{\circ}\text{C}}=77$, $N_{10-25^{\circ}\text{C}}=77$, $N_{15-30^{\circ}\text{C}}=76$, and $N_{20-35^{\circ}\text{C}}=78$. For each group, brood cells were placed into a plastic box (20 x 20 x 6 cm) which was partly covered with plastic, partly with gauze to allow for ventilation. The plastic boxes were put into a cardboard box to prevent desiccation. The box of the control group was stored in the dark in the observation hut where the bees nested. The boxes of the treatment groups were stored in dark climate chambers (Ehret ATS 1373, Emmendingen, Germany, +/- 1°C for constant temperatures; Binder KB 115, Tuttlingen, Germany, for fluctuating temperature regimes). Temperatures were monitored using thermobuttons (iButton DS1921G, Maxim Integrated Products, USA). All brood cells were daily checked for developmental stage and mortality of bee progeny.

We defined four visibly distinguishable developmental phases until the cocoon was finished: 1) egg phase (from egg laying to the beginning of feeding), 2) early consumption phase (from the beginning of feeding to the beginning of defecation), 3) defecation phase (from the beginning of defecation to the beginning of cocoon spinning), and 4) cocooning (from the beginning of cocoon spinning to the completely darkened cocoon). Ten to fourteen days after cocoon completion, individual brood cells were opened, the cocoon was cleaned of faeces, weighed to the nearest 0.1 mg, placed in a new trap nest in the cardboard box, and put back in the according conditions. The weight of the cocoon with the prepupa inside is further referred to as “prepupal weight”.

2.3.4 Observation cocoons

Since the cocoons of *O. bicornis* are opaque, we could not easily observe the development inside the cocoon. In order to check development inside the cocoon without using the X-ray method (Stephen and Undurraga 1976), we cut a hole in the upper side of the cocoon (at least one week after cocoon completion to prevent spinning again and closing the spyhole). The holes were sealed with a transparent foil fixed with odourless superglue (UHU® Sekundenkleber, SUPERFLEX GEL). The spyhole allowed the daily monitoring of development inside the cocoon and the exact determination of the date of pupation and adult eclosion.

A sample of observation cocoons for every temperature treatment was prepared at the end of June 2008 ($N_{\text{Hut}}=11$, $N_{17.5^{\circ}\text{C}}=10$, $N_{22.5^{\circ}\text{C}}=11$, $N_{27.5^{\circ}\text{C}}=15$, $N_{10-25^{\circ}\text{C}}=10$, $N_{15-30^{\circ}\text{C}}=15$, and $N_{20-35^{\circ}\text{C}}=15$). Since some bees were already in the pupal stage at this time, their exact date of pupation could not be recorded. Such observation cocoons were excluded from all analyses except in the 15-30°C-group, where they were included for the calculation of the duration of complete development. The duration of the prepupal and pupal stage had to be estimated based on only two individuals in this group, since these bees entered the pupal stage unexpectedly early.

2.3.5 Wintering

All cocoons were transferred to a climate chamber with constant 4°C on 2 October 2008. On 18 March 2009, the cocoons were placed in transparent plastic containers (with gauze-covered windows for ventilation) and transferred to the hut where they experienced the

natural temperature increase and emerged in spring. In the middle of May, all failed cocoons were opened and inspected for mortality stage. Some cocoons/bees from each temperature treatment were used for other investigations during wintering, therefore we used the remaining cocoon number as reference (=100%) for the determination of mortality for the stages inside the cocoon.

2.3.6 Data analyses

Since the body size of *O. bicornis* depends largely on the provision weight (Radmacher and Strohm 2010), we used the ratio of prepupal weight to provision weight as a measure for body size to correct for individual differences in provision weight. ANOVAs (PASW Statistics 18 (i.e. SPSS 18.0)) with temperature level (cool, medium, and warm) and temperature mode (constant and fluctuating) as fixed factors were used to test for effects of temperature level and mode on the duration of all recorded developmental stages, on the duration of complete development, and on the prepupal weight. The control group (Hut) was not included in the statistical analyses, but is presented in the figures as a reference for development under natural conditions. Mortality rates were analysed with logistic regressions with survival status (dead or alive) in the respective stage as dependent variable and temperature level as well as temperature mode (categorized) as covariates (PASW Statistics 18).

2.4 RESULTS

2.4.1 Prepupal weight

There was a significant effect of temperature level and temperature mode on the ratio of prepupal weight to provision weight (temperature level: $F=137.8$, $df=2$, $p<0.001$; temperature mode: $F=325.8$, $df=1$, $p<0.001$). Higher temperatures resulted in lower prepupal weights and fluctuating temperatures tended to result in higher prepupal weights than the constant mean temperatures (Fig. 2.1). There was a significant interaction between temperature level and mode indicating that the decrease in weight ratio with increasing temperatures was smaller under fluctuating conditions (Fig. 2.1; temperature level x temperature mode: $F=69.9$, $df=2$, $p<0.001$). Concerning the constant temperatures, the 22.5°C treatment deviated from the general trend in that the prepupal weights were comparatively (and unusually) low.

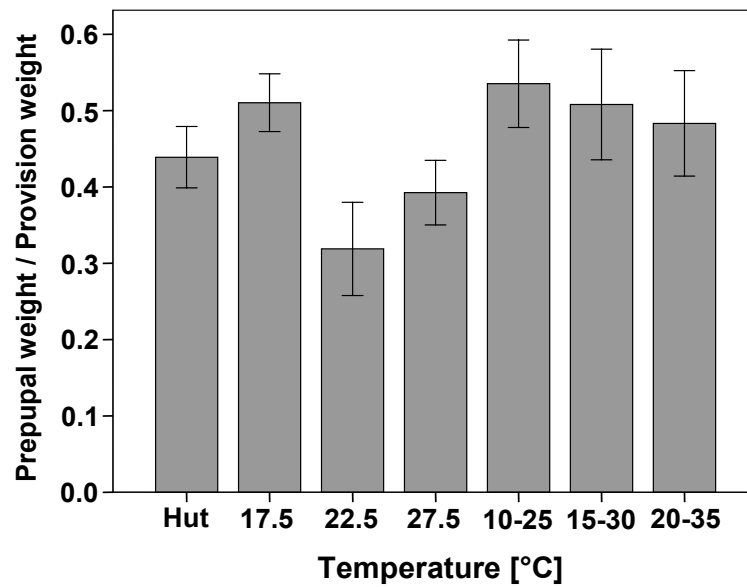


Figure 2.1: Ratio of prepupal weight to provision weight (mean \pm SD) as a measure for body size of *O. bicornis* offspring reared at three constant ($N_{17.5^{\circ}\text{C}} = 70$, $N_{22.5^{\circ}\text{C}} = 67$, $N_{27.5^{\circ}\text{C}} = 74$) and three fluctuating ($N_{10-25^{\circ}\text{C}} = 73$, $N_{15-30^{\circ}\text{C}} = 66$, $N_{20-35^{\circ}\text{C}} = 74$) temperature regimes. The control group ($N_{\text{Hut}} = 58$) was exposed to natural temperature conditions.

Table 2.1: Mortality rates of *O. bicornis* offspring reared at seven different temperature treatments (three constant temperatures: 17.5, 22.5, and 27.5°C; three fluctuating temperature regimes: 10-25, 15-30, and 20-35°C; Hut = natural temperature conditions). Percentage values refer to the given sample sizes of brood cells or cocoons, respectively.

Treatment	Brood cells N	Egg-cocoon mortality [%]	Cocoons N	Prepupa mortality [%]	Pupa mortality [%]	Adult mortality [%]
Hut	66	9.1	46	0	0	0
17.5°C	77	9.1	56	1.8	0	16.1
22.5°C	77	13.0	53	0	1.9	24.5
27.5°C	77	2.4	74	0	0	100.0
10-25°C	77	5.2	59	3.4	0	0
15-30°C	76	10.5	52	26.9	3.8	1.9
20-35°C	78	5.2	65	9.2	1.5	86.2

2.4.2 Mortality

The mortality data are given in Table 2.1. Egg to cocoon mortality and pupal mortality were neither influenced by temperature level (egg to cocoon: Wald=1.728, df=1, p=0.189; pupa: Wald= 0.227, df=1, p=0.634) nor by temperature mode (egg to cocoon: Wald=0.125, df=1, p=0.724; pupa: Wald=1.014, df=1, p=0.314). Prepupal mortality was affected by temperature mode (Wald=10.103, df=1, p=0.001), but not by temperature level (Wald=0.324, df=1, p=0.569) with unusually high mortality in 15-30°C. Adult mortality was very high in the warm treatments (27.5 and 20-35°C) and significantly influenced by both temperature level (Wald=94.511, df=1, p<0.001) and temperature mode (Wald=22.18, df=1, p<0.001).

2.4.3 Duration of development

Generally, the duration of all four developmental phases until cocoon completion decreased with increasing temperature, except for the defecation phase in 22.5°C that was anomalously long (Fig. 2.2; egg phase: F=1530.9, df=2, p<0.001; early consumption phase: F=1779.1, df=2, p<0.001, defecation phase: F=246.02, df=2, p<0.001; cocooning: F=66.7, df=2 p<0.001). Fluctuating (vs. constant) conditions had a significant accelerating effect on the early consumption phase (F=27.6, df=1, p<0.001), the defecation phase (F=936.2, df=1, p<0.001), and cocooning (F=16.84, df=1, p<0.001), but not on the egg phase (F=2.99, df=1, p=0.085).

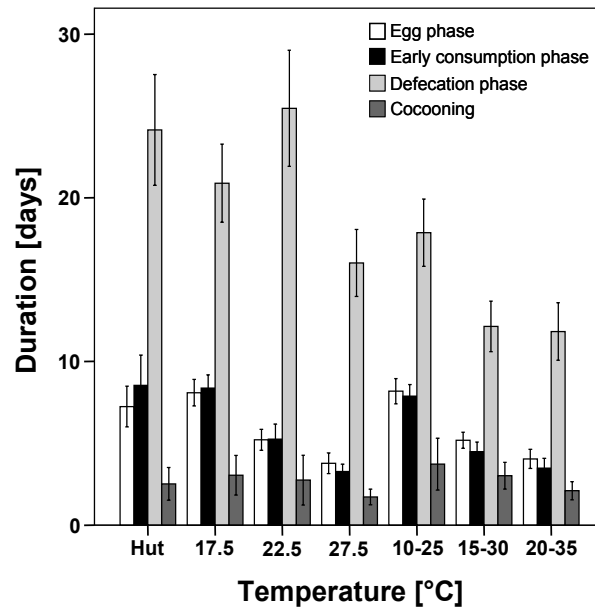


Figure 2.2: Duration (mean \pm SD) of four visibly distinguishable developmental phases (egg phase, early consumption phase, defecation phase, and cocooning) from egg to cocoon completion of *O. bicornis* offspring reared at seven different temperature regimes (control: $N_{\text{Hut}} = 58$; constant temperatures: $N_{17.5^\circ\text{C}} = 70$, $N_{22.5^\circ\text{C}} = 67$, $N_{27.5^\circ\text{C}} = 74$; fluctuating temperature regimes: $N_{10-25^\circ\text{C}} = 73$, $N_{15-30^\circ\text{C}} = 66$, $N_{20-35^\circ\text{C}} = 74$).

The duration of the prepupal stage was significantly influenced by temperature level ($F=17.37$, $df=2$, $p<0.001$) and temperature mode ($F=16.54$, $df=1$, $p<0.001$). While the accelerating effect of fluctuating (vs. constant) temperatures followed the general pattern found in the other developmental stages, the observed effect of temperature level was converse to all other investigated developmental stages since the duration of the prepupal stage increased considerably in the warm treatments (Fig. 2.3). Consistent with the general pattern, the duration of the pupal stage decreased with increasing temperature ($F=157$, $df=2$, $p<0.001$), but there was no significant effect of temperature mode ($F=3.489$, $df=1$, $p=0.069$) in this stage (Fig. 2.3).

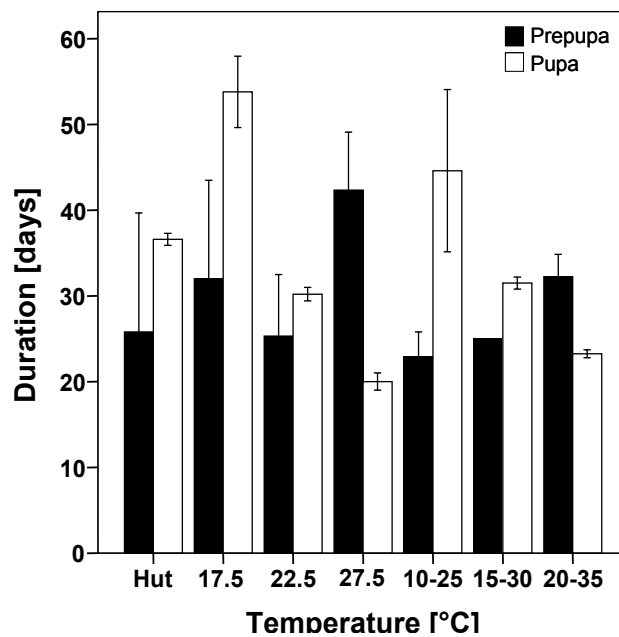


Figure 2.3: Duration (mean \pm SD) of the prepupal and the pupal stage of *O. bicornis* offspring reared at seven different temperature regimes (control: $N_{\text{Hut}} = 10$; constant temperatures: $N_{17.5^{\circ}\text{C}} = 10$, $N_{22.5^{\circ}\text{C}} = 10$, $N_{27.5^{\circ}\text{C}} = 9$; fluctuating temperature regimes: $N_{10-25^{\circ}\text{C}} = 10$, $N_{15-30^{\circ}\text{C}} = 2$, $N_{20-35^{\circ}\text{C}} = 8$).

We found a significant effect of temperature level ($F=73.9$, $df=2$, $p<0.001$) and temperature mode ($F=42.43$, $df=1$, $p<0.001$) on the duration of complete development from egg to adult eclosion. There was a general decrease in duration with increasing temperature, however, the difference between the medium and the warm temperature treatments was small (Fig. 2.4). Fluctuating (vs. constant) conditions led to an acceleration of development. Though no significant interaction of temperature level and temperature mode was detected ($F=1.134$, $df=2$, $p=0.33$), the accelerating effect of fluctuating conditions tended to decrease with increasing temperature (Fig. 2.4).

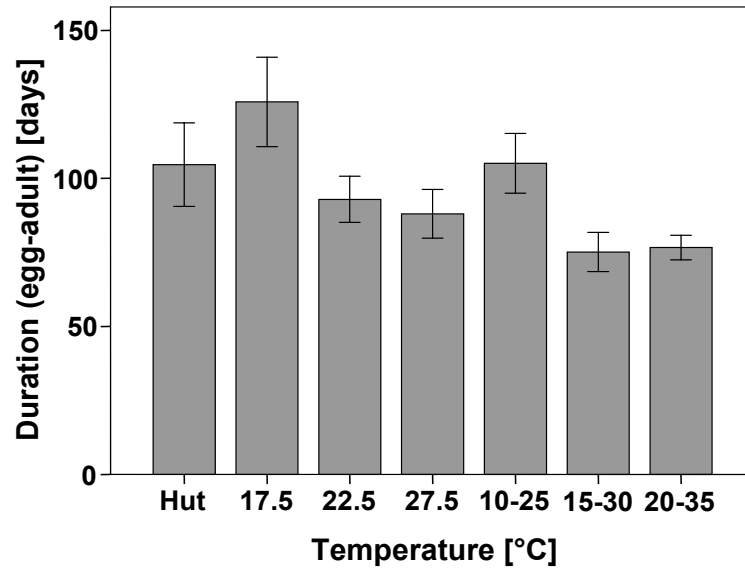


Figure 2.4: Duration (mean \pm SD) of complete development (from egg to adult eclosion) of *O. bicornis* offspring reared at seven different temperature regimes (control: $N_{\text{Hut}} = 10$; constant temperatures: $N_{17.5^{\circ}\text{C}} = 10$, $N_{22.5^{\circ}\text{C}} = 10$, $N_{27.5^{\circ}\text{C}} = 9$; fluctuating temperature regimes: $N_{10-25^{\circ}\text{C}} = 10$, $N_{15-30^{\circ}\text{C}} = 9$, $N_{20-35^{\circ}\text{C}} = 8$).

2.5 DISCUSSION

2.5.1 Prepupal weight

In general, our results on the effects of temperature on prepupal weight are consistent with the temperature-size rule (Atkinson 1994) and a previous study on *O. bicornis* which revealed a strong decrease in body size with increasing constant rearing temperatures (Radmacher and Strohm 2010). However, bee offspring in the 22.5°C treatment had an unexpectedly low prepupal weight. Taking into account a comparatively frequent occurrence of pollen remnants in the brood cells and the anomalously long defecation phase, the conditions might have been somehow suboptimal in this treatment. We were not able to explain this finding since the 22.5°C treatment was treated in the same way as the others. Regarding the 17.5 and 27.5°C treatment, the decrease in body weight was not as pronounced as in a previous study (Radmacher and Strohm 2010), where overall warmer temperatures (20, 25, and 30°C) were used and the weight of adult bees inside their cocoons was determined.

Under fluctuating conditions, the bee offspring attained higher prepupal weights than in the respective constant mean temperatures and the decrease in prepupal weight with increasing temperature was considerably smaller than in the constant treatments. These findings support

the view that the results of studies conducted with constant temperatures in the laboratory can hardly be extrapolated to populations living under natural fluctuating conditions (Beck 1983; Ratte 1984). Moreover, our results suggest that under fluctuating conditions an overall warming of several degrees during development would probably have only a small negative effect on the prepupal weight in *O. bicornis*. However, weight loss can be considerable in the pupal, pre-wintering, and wintering phases (Bosch and Vicens 2002; Sgolastra et al. 2010). Therefore, warming during the entire life cycle could have stronger effects on final body weight.

2.5.2 Mortality

The rates of mortality from egg to cocoon and in the pupal stage were not influenced by temperature level or temperature mode and they were within the usual range for this species (see the Hut group Tab. 2.1; Strohm et al. 2002). We suppose that the significant effect of temperature mode on prepupal mortality and the comparatively high prepupal mortality in the 15-30°C treatment were probably caused by a mould infestation that apparently occurred only in the 15-30°C and 20-35°C treatment at the beginning of June 2008. Therefore, we assume that mortality until the adult stage was not severely affected by developmental temperature.

In contrast, mortality in the adult stage increased considerably with increasing temperature. Though mortality rates were generally lower under fluctuating than under constant temperature conditions, adult mortality was very high in the 20-35°C treatment. Since all bees were transferred to 4°C when the slowest group (17.5°C) had completed their development, particularly the bees in the warm treatments experienced a long pre-wintering period (about 60-70 days) in high temperatures that probably caused the very high adult mortality due to fat body depletion (Bosch et al. 2000, 2010; Bosch and Kemp 2004). The length of the pre-wintering period was similar for the bees in the warm and in the medium temperature treatments; however, the bees in the medium treatments were exposed to lower temperatures during this time. The medium temperatures seemed to be less harmful than the warm temperatures, particularly under fluctuating conditions. For comparison, bees under natural conditions (Hut) experienced a pre-wintering period of 46 days on average in $17.0 \pm 7.8^\circ\text{C}$ (mean \pm SD). The elevated adult mortality in the 17.5°C treatment (16.1 %; compared to 0 % in 10-25°C and in the control) was possibly caused by a too short pre-wintering period. Since the 17.5°C group was transferred to 4°C shortly after we had observed the last

adult eclosion, the pre-wintering period might have been too short for some individuals to lower the respiration rate to an appropriate level for wintering (Bosch et al. 2010).

Our results for adult mortality underlined the importance of the duration of and the temperature during the pre-wintering phase for the survival and fitness of *Osmia* bees (Bosch and Kemp 2004). However, even with ongoing climate change it seems unlikely that in the foreseeable future natural *O. bicornis*-populations will be exposed to such warm pre-wintering temperatures as we used for our warm treatments. Due to the low adult mortality in the fluctuating 15-30°C treatment, we tentatively suggest that climate change would probably not have a strong negative effect on the survival of *O. bicornis* during the pre-wintering phase.

2.5.3 Duration of development

For most (but not all) of the recorded developmental stages and the complete development, we found a significant acceleration in the fluctuating versus the corresponding constant temperature treatments. Regarding complete development, the accelerating effect of temperature fluctuations tended to decrease with increasing temperature, even though this trend was not significant. A similar pattern was found in mosquitoes (Joshi 1996). These findings are generally consistent with the often observed pattern that for a certain insect species temperature fluctuations in the range below a certain temperature may cause an acceleration of development, while the converse is true for fluctuations near the temperature optimum (Beck 1983; Hagstrum and Milliken 1991). However, not all insect species follow this pattern (see e.g. Neumann and Heimbach 1975; Welbers 1975). While we did not detect a deceleration due to fluctuating temperatures in our warm treatments, such an effect might occur at temperatures higher than used in this study. The congeneric *O. lignaria* developed faster in two fluctuating treatments (14:27°C and orchard) than in equivalent constant temperatures (22 and 18°C, respectively; Bosch and Kemp 2000), but no warmer fluctuating regime was investigated.

Development time of ectotherms typically decreases with increasing temperature (up to an optimal temperature) due to the acceleration of biochemical processes at higher temperatures (Ratte 1984; Atkinson 1996). However, the effect of temperature on development time has been shown to differ among species, populations, and developmental stages of a species (Ratte 1984; Ayres and Scriber 1994; Bosch and Kemp 2000; Rombough 2003; Kemp and

Bosch 2005; Folguera et al. 2010). We found the typical decrease of development time with increasing temperatures in almost all investigated developmental stages of *O. bicornis* – except in the prepupal stage whose duration increased in the warm treatments.

Based only on the data in this study, we cannot completely exclude that this extension of the prepupal stage could be a sign of harmful but not yet lethal temperature. However, *Osmia* bees probably undergo a summer diapause in the prepupal stage (Kemp et al. 2004; Bosch et al. 2010) that seems to be the developmental stage with the highest plasticity with regard to duration and with a lower susceptibility to warm temperatures than the adult stage (Sgolastra 2007; Bosch and Kemp 2000; Bosch et al. 2000, 2010). Our results are consistent with Kemp and Bosch (2005) who found a U-shaped thermal response of the prepupal stage in *O. lignaria* with increasing duration below and above a certain medium temperature and we assent to their assumption that prolonging the prepupal phase could be a suitable mechanism to adjust adult eclosion date to the onset of colder temperatures in autumn if development was accelerated due to hot summers (Kemp and Bosch 2005; Bosch et al. 2010). This adjustment seems to be very important to avoid long pre-wintering periods with their negative effects on survival and fitness (Bosch and Kemp 2004; Bosch et al. 2010; and see 2.5.2) The similar duration of complete development in the medium and warm treatments (Fig. 2.4) suggests that the acceleration of the larval and pupal development due to high temperatures was largely compensated by the prolonged prepupal phase. Whether the observed extension of the prepupal period was triggered by the actual temperature in the prepupal phase or by the temperature experienced earlier in development can not be decided based on our data (but see Kemp and Bosch 2005). However, the observed developmental plasticity in the duration of the prepupal stage might help *O. bicornis* to cope with the increasing frequency of heat waves in summer and the overall elevated temperatures due to climate change.

2.6 ACKNOWLEDGEMENTS

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CHAPTER 3

TEMPERATURE EFFECTS ON SOLITARY BEE DEVELOPMENT – DOES TEMPERATURE EXPERIENCED AS LARVA INFLUENCE THE DURATION OF THE PREPUPAL STAGE?

Apidologie, submitted

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3.1 SUMMARY

Increasing temperatures usually accelerate the development of insects. Recently, we found that the prepupal stage in *Osmia bicornis* offspring was prolonged when exposed to high temperatures for their entire development. Here we tested whether this effect is directly triggered by the temperature during the prepupal stage. We kept larvae at low temperatures until cocoon completion and allocated them to three constant and three fluctuating temperature regimes for the subsequent development. The duration of all stages including the prepupal phase decreased with increasing temperature. Thus, contrary to our expectation the duration of the prepupal stage in *O. bicornis* is not only controlled by the actual temperature but seems rather to be influenced by the temperature experienced during previous stages. The extension of the prepupal phase might be a mechanism to adjust the adult eclosion to the onset of wintering temperatures when larval development was accelerated due to hot summers.

3.2 INTRODUCTION

For ectotherms, temperature is one of the most important environmental factors and the temperature experienced during development strongly affects morphology and many life history traits (Steigenga and Fischer 2009), e.g. body size (Atkinson 1994), development time (Ratte 1984), and fecundity (e.g. Stillwell and Fox 2005). Many studies have investigated different effects of developmental temperature in a variety of ectotherms, particularly in aquatic animals and in insects, including bees (e.g. Ratte 1984; Pepin 1991; Bosch and Kemp 2000; Kemp and Bosch 2000; Medrzycki et al. 2010; Radmacher and Strohm 2010, 2011a; Stoner et al. 2010). In the majority of these studies, offspring of the study species were exposed to one of several temperature treatments for their entire development. However, temperature effects on development time are known to differ among developmental stages (Li and Jackson 1996; Petersen et al. 2000; Rombough 2003; Folguera et al. 2010).

Recently, we found in the Red mason bee *Osmia bicornis* (Linnaeus 1758, Hymenoptera, Megachilidae; formerly *O. rufa*) that - despite the usual acceleration of development due to higher temperatures - the prepupal phase was prolonged in warm compared to medium and cool temperature treatments (Radmacher and Strohm 2011a). The solitary, univoltine, and spring-active *Osmia* bees develop during summer, eclose as adults in autumn, winter inside their cocoons and emerge the next spring. For these bees, the duration of the pre-wintering period, i.e. the period from adult eclosion to the onset of wintering temperatures, seems to be crucial for overwintering survival since long pre-wintering periods as adults cause a depletion of fat reserves and increased mortality (Bosch et al. 2000, 2010; Bosch and Kemp 2004; Radmacher and Strohm 2011a). Since the timing of adult eclosion seems to depend largely on the duration of the prepupal period (Sgolastra 2007), the observed extension of this phase in *O. bicornis* might be an adaptive mechanism to avoid the negative effects of a long pre-wintering period (Bosch et al. 2000, 2010; Kemp and Bosch 2005; Radmacher and Strohm 2011a). In our previous study, we had exposed the *O. bicornis* offspring to the experimental temperatures for their entire development. Thus, the observed extension of the prepupal phase might have been triggered by the actual temperature in the prepupal phase (as it was shown for *O. lignaria* by Kemp and Bosch 2005) or by the temperature experienced earlier in development.

To test whether the duration of the prepupal stage is only controlled by the actual prevailing temperature in *O. bicornis*, we decoupled temperature influences on larval development and the development inside the cocoon (prepupal and pupal stage) and monitored the development of the bee progeny. We kept the temperature until cocoon spinning on a cool level and provided three different temperature levels (cool, medium, and warm) for subsequent development. Additionally, we examined whether fluctuating temperatures differed in their effect from the corresponding constant temperatures. According to the results for *O. lignaria* (Kemp and Bosch 2005) that suggest that the extension of the prepupal phase (as a response to warm temperatures) is controlled by the actually experienced temperature, we expected that the duration of the prepupal stage should increase in the warm treatments. However, if the duration of the prepupal phase decreased with increasing temperature, this finding – combined with the results of our previous study (Radmacher and Strohm 2011a) - would support the alternative hypothesis that the duration of the prepupal phase is influenced by the temperature experienced in previous stages.

3.3 MATERIALS AND METHODS

3.3.1 Bees

O. bicornis (Hymenoptera, Megachilidae) is a common and abundant solitary bee in Central Europe that readily accepts trap nests. The flight season of this polylectic species usually lasts from early April to mid-June. Brood cells were obtained from a free-flying *O. bicornis* population nesting in trap nests in an observation hut near the botanical garden of the University of Regensburg (for more details about the trap nests see Strohm et al. 2002; Radmacher and Strohm 2011a). During the peak nesting season in 2009 (14 to 26 April), we used 463 brood cells with one to three day old eggs from 50 nests for our investigations. After cutting of a brood cell from the nest, it was brought into the laboratory where mass of the provisions (with the attached egg) was determined to the nearest 0.1 mg (Sartorius analytic A120 S) and put back into the brood cells (for details see Radmacher and Strohm 2010, 2011a). The brood cells were resealed with paper on the side and transparent foil on the top (fixed with adhesive tape) and randomly allocated to the two initial temperature treatments (T1, see below).

3.3.2 Temperature treatments

In this study, we wanted to decouple temperature effects on larval development and later developmental stages with particular attention to effects on the prepupal and pupal stage. For the development from egg until cocoon completion, the brood cells were apportioned between two temperature treatments - constant 17.5°C (N=234) and fluctuating 10-25°C (N=229), (called “T1” in the following for “Temperature treatment 1”). We chose these two treatments based on our previous study (Radmacher and Strohm 2011a) where these conditions were appropriate for bee development. We employed a constant as well as a fluctuating treatment to cover possible effects of different patterns of temperature regimes.

The brood cells were daily checked for developmental stage and mortality of bee progeny. We defined four clearly distinguishable developmental phases from egg until cocoon completion: 1) egg phase (from egg laying to the beginning of feeding), 2) early consumption phase (from the beginning of feeding to the beginning of defecation), 3) defecation phase (from the beginning of defecation to the beginning of cocoon spinning), and 4) cocooning (from the beginning of cocoon spinning to the completely darkened cocoon).

On the day of cocoon completion, individual brood cells were opened, the cocoon was cleaned of faeces, weighed to the nearest 0.1 mg (Sartorius analytic A120 S), and placed in a new trap nest. The weight of the cocoon with the prepupa inside is further referred to as “prepupal weight”. After cocoon completion, the individuals were randomly allocated to seven temperature treatments for the subsequent development (called “T2” in the following). We used the same seven temperature treatments as in Radmacher and Strohm (2011a), i.e. three fluctuating temperature regimes (10-25, 15-30, and 20-35°C), three constant temperatures that represented the mean temperatures of the fluctuating regimes (17.5, 22.5, and 27.5°C), and one control group (“Hut”) that was exposed to natural temperature conditions. The sample sizes were: $N_{\text{Hut}}=59$, $N_{17.5^{\circ}\text{C}}=59$, $N_{22.5^{\circ}\text{C}}=59$, $N_{27.5^{\circ}\text{C}}=58$, $N_{10-25^{\circ}\text{C}}=59$, $N_{15-30^{\circ}\text{C}}=59$ and $N_{20-35^{\circ}\text{C}}=58$. In the three fluctuating regimes, temperature followed a sine curve and reached the eponymous minimum and maximum values once in 24 h. For more details about the temperature treatments see Radmacher and Strohm (2011a).

3.3.3 Observation cocoons

The cocoons of *O. bicornis* are opaque and the X-ray method (Stephen and Undurraga 1976) for the monitoring of bee development inside the cocoon was not applicable for us.

Therefore, we cut a spyhole in the upper side of the cocoon that was immediately resealed with transparent foil, fixed with odourless superglue (see Radmacher and Strohm (2011a) for details). We prepared a sample of observation cocoons for each temperature treatment (T2) in June 2009 ($N_{\text{Hut}}=12$, $N_{17.5^{\circ}\text{C}}=13$, $N_{22.5^{\circ}\text{C}}=13$, $N_{27.5^{\circ}\text{C}}=15$, $N_{10-25^{\circ}\text{C}}=12$, $N_{15-30^{\circ}\text{C}}=14$ and $N_{20-35^{\circ}\text{C}}=13$). About one half of these observation cocoons for each treatment had experienced T1 of either 17.5°C or $10-25^{\circ}\text{C}$. By daily monitoring the developmental stage, the exact date of pupation and adult eclosion was determined. For data analyses, we used only cocoons with complete data sets, excluding bee offspring that died during development or that were already in the pupal stage at the preparation of the spyhole.

3.3.4 Data analyses

Since the body size of *O. bicornis* largely depends on the provision weight (Radmacher and Strohm 2010), we used the ratio of prepupal weight to provision weight as a measure for body size to correct for individual differences in provision weight. The body size and the duration of all recorded developmental phases until cocoon completion were compared between the constant and the fluctuating T1-treatment using t-tests (PASW Statistics 18, i.e. SPSS 18.0). Average values are given as mean \pm SD. Differences between treatment groups with regard to mortality from egg to cocoon and the frequency of occurrence of pollen remnants in the brood cells were analysed using χ^2 -tests with Yates correction (BiAS 8.2). ANOVAs (PASW Statistics 18) with T1 (17.5 vs. $10-25^{\circ}\text{C}$), T2-level (cool, medium, and warm), and T2-mode (constant vs. fluctuating) as fixed factors were conducted to investigate temperature effects on the duration of (1) the prepupal stage, (2) the pupal stage, and (3) the complete development from egg to adult. Due to small violations of the assumption of homoscedasticity, the level of significance for the ANOVAs was lowered to $\alpha=0.01$ (Bühl and Zöfel 2005). The control group (Hut) was not included in the statistical analyses, but is presented in the figures as a reference for development under natural conditions.

3.4 RESULTS

3.4.1 Development from egg to cocoon

Bee larvae that developed at a constant T1 of 17.5°C needed significantly more time for the development from egg until cocoon completion (57.6 ± 4.6 days) than those in the fluctuating $10-25^{\circ}\text{C}$ treatment (40.5 ± 3.6 days; $T=41.82$, $df=391.8$, $p<0.001$). This remarkable difference

in development time was mainly due to the considerably longer defecation phase in 17.5°C (Fig. 3.1; $T=42.03$, $df=279.3$, $p<0.001$). In addition, all other recorded phases were slightly but also significantly longer in 17.5°C than in 10-25°C (Fig. 3.1; egg phase: $T=3.982$, $df=409$, $p<0.001$; early consumption phase: $T=24.71$, $df=389.2$, $p<0.001$; cocooning: $T=2.457$, $df=402.1$, $p=0.014$).

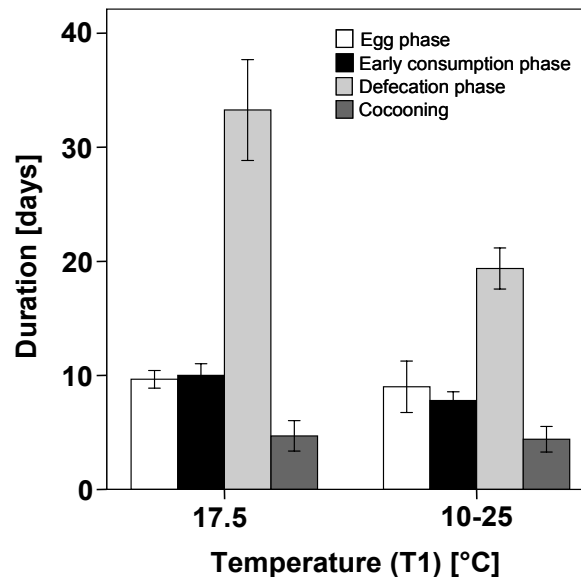


Figure 3.1: Duration (mean \pm SD) of four visibly distinguishable developmental phases (egg phase, early consumption phase, defecation phase, and cocooning) from egg to cocoon completion of *O. bicornis* offspring reared at a constant (17.5°C, $N=210$) and a fluctuating (10-25°C, $N=201$) temperature regime.

Body size – represented by the ratio of prepupal weight to provision weight – differed significantly between the two T1-treatments ($T=25.11$, $df=398.1$, $p<0.001$). Bee offspring in 17.5°C attained a significantly lower prepupal weight (prepupal weight/provision weight = 0.378 ± 0.053) than in 10-25°C (0.501 ± 0.047). The frequency of brood cells with pollen remnants was significantly higher in 17.5°C than in 10-25°C (10 % vs. 1.5 %, respectively; $\chi^2=12.016$, $p=0.0005$). However, there was no significant difference in mortality rate of the larvae (17.5°C: 10.3 %; 10-25°C: 12.3 %; $\chi^2=0.2748$, $p=0.600$).

3.4.2 Development inside the cocoon

We found a significant effect of T1 ($F=98.026$, $df=1$, $p<0.001$) on the duration of the prepupal stage. In all seven T2-treatments, bee offspring that were exposed to T1 of 17.5°C during larval development stayed longer in the prepupal stage than bee larvae that had developed in 10-25°C (Fig. 3.2). The absolute differences in the duration of the prepupal phase between

the two T1-treatments were somewhat larger in the cool than in the medium and warm T2-treatments (interaction T1xT2-level: $F=5.965$, $df=2$, $p=0.004$; Fig. 3.2). There was no significant effect of T2-mode ($F=1.452$, $df=1$, $p=0.233$) on the duration of the prepupal stage. However, with increasing T2-level the duration of the prepupal stage significantly decreased ($F=543.481$, $df=2$, $p<0.001$; Fig. 3.2). The duration of the pupal stage was not significantly affected by T1 ($F=4.435$, $df=1$, $p=0.039$) or T2-mode ($F<0.001$, $df=1$, $p=0.998$), but decreased significantly with T2-level ($F=2686.2$, $df=2$, $p<0.001$; Fig. 3.3).

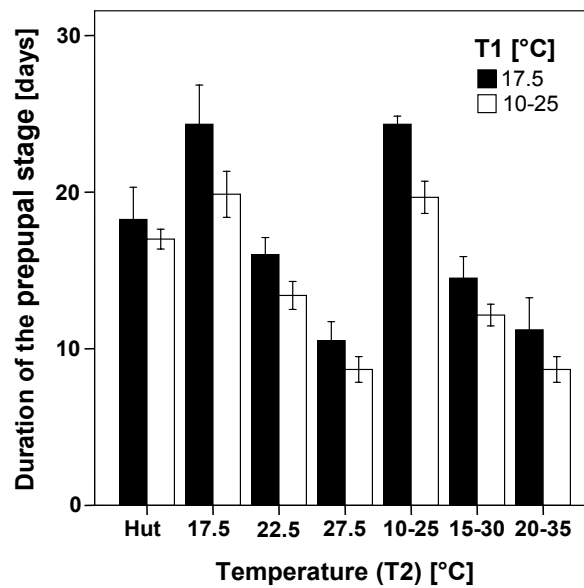


Figure 3.2: Duration (mean \pm SD) of the prepupal stage of *O. bicornis* offspring reared at a constant (17.5°C) and a fluctuating (10-25°C) temperature regime until cocoon completion (T1) and at seven different temperature regimes for the development inside the cocoon (T2; Sample sizes: T1=17.5°C/10-25°C: $N_{\text{Hut}} = 4/6$, $N_{17.5^\circ\text{C}} = 6/7$, $N_{22.5^\circ\text{C}} = 6/5$, $N_{27.5^\circ\text{C}} = 6/6$, $N_{10-25^\circ\text{C}} = 6/6$, $N_{15-30^\circ\text{C}} = 6/7$, $N_{20-35^\circ\text{C}} = 5/6$).

3.4.3 Complete development

The duration of complete development from egg to adult was significantly influenced by T1 ($F=1109.55$, $df=1$, $p<0.001$) and T2-level ($F=1300.36$, $df=2$, $p<0.001$), but not T2-mode ($F=5.038$, $df=1$, $p=0.029$). The effect of T1 on the duration of the larval and the prepupal stages resulted in an overall longer development of the bees that experienced constant 17.5°C compared to bees that were exposed to fluctuating 10-25°C during larval development (Fig. 3.4). The effect of T2-level on the duration of the prepupal and the pupal stage resulted in a significant decrease in the duration of the complete development with increasing temperature in the constant and fluctuating treatments (Fig. 3.4).

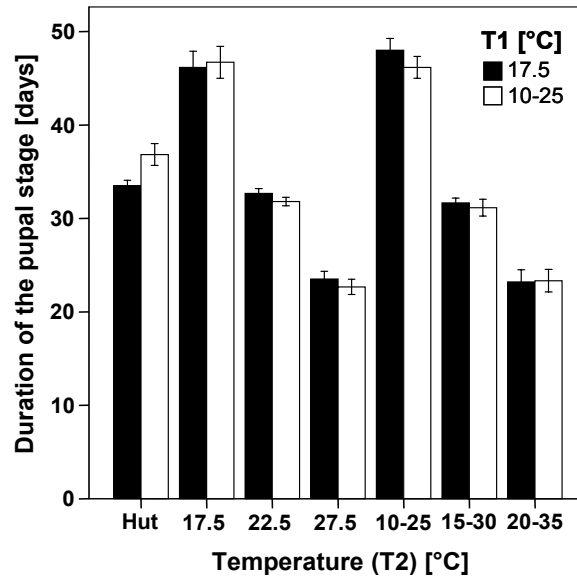


Figure 3.3: Duration (mean \pm SD) of the pupal stage of *O. bicornis* offspring reared at a constant (17.5°C) and a fluctuating (10-25°C) temperature regime until cocoon completion (T1) and at seven different temperature regimes for the development inside the cocoon (T2; Sample sizes: T1=17.5°C/10-25°C: $N_{\text{Hut}} = 4/6$, $N_{17.5^\circ\text{C}} = 6/7$, $N_{22.5^\circ\text{C}} = 6/5$, $N_{27.5^\circ\text{C}} = 6/6$, $N_{10-25^\circ\text{C}} = 6/6$, $N_{15-30^\circ\text{C}} = 6/7$, $N_{20-35^\circ\text{C}} = 5/6$).

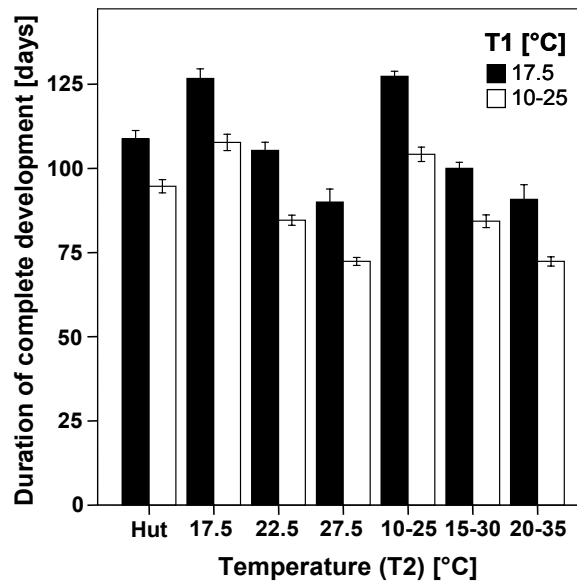


Figure 3.4: Duration (mean \pm SD) of the complete development (from egg to adult eclosion) of *O. bicornis* offspring reared at a constant (17.5°C) and a fluctuating (10-25°C) temperature regime until cocoon completion (T1) and at seven different temperature regimes for the development inside the cocoon (T2; Sample sizes: T1=17.5°C/10-25°C: $N_{\text{Hut}} = 4/6$, $N_{17.5^\circ\text{C}} = 6/7$, $N_{22.5^\circ\text{C}} = 6/5$, $N_{27.5^\circ\text{C}} = 6/6$, $N_{10-25^\circ\text{C}} = 6/6$, $N_{15-30^\circ\text{C}} = 6/7$, $N_{20-35^\circ\text{C}} = 5/6$).

3.5 DISCUSSION

3.5.1 Development from egg to cocoon

All four recorded developmental phases and the complete development until cocoon completion were significantly shorter when larvae were exposed to fluctuating T1-temperatures (10-25°C) as opposed to the constant mean temperature (17.5°C). The development of insects is often accelerated by fluctuating temperatures compared to the respective constant mean temperature, particularly in lower temperature regimes (Beck 1983; Ratte 1984; Hagstrum and Milliken 1991). This was also shown for *Osmia* bees (Bosch and Kemp 2000; Radmacher and Strohm 2011a).

However, the magnitude of the difference in the duration of development until cocoon completion between the fluctuating and constant T1 in the present study (about 17 days) was unexpected. In our previous study, the duration of development until cocoon completion was more similar in these treatments (17.5°C: 40.4 ± 2.5 days; 10-25°C: 37.6 ± 2.6 days; Radmacher and Strohm 2011a). This difference was mainly due to a longer defecation phase of larvae in 17.5°C in the present study (13 days longer than in Radmacher and Strohm (2011a)). The considerable proportion of larvae that did not consume all their provisions and the comparatively low prepupal weight suggest that the conditions in the 17.5°C treatment were somehow suboptimal during this study. We were not able to explain this finding. Olifir (1989) reported a vital range of 18-32°C for the larvae of *O. bicornis*, however, the 17.5°C treatment was suitable for the bee offspring in our previous study (Radmacher and Strohm 2011a) and we used exactly the same methods and climate chambers. Moreover, mortality rates were similar and in a usual range for this species (Strohm et al. 2002; Radmacher and Strohm 2011a) in both T1 treatments.

3.5.2 Development inside the cocoon and complete development

In contrast to larval development, we detected no significant effect of T2-mode (constant vs. fluctuating temperatures) on the duration of the prepupal stage, the pupal stage, and complete development from egg to adult eclosion. Radmacher and Strohm (2011a) found a significant effect of temperature mode on the duration of the prepupal stage and complete development (but not on the duration of the pupal stage) and Bosch and Kemp (2000) reported a remarkable reduction in the duration of the prepupal and the pupal stage in fluctuating (14-27°C) compared to the corresponding constant (22°C) temperatures in *O. lignaria*. However,

in these studies, the *Osmia* offspring were exposed to the different temperature treatments during their entire development. That T2-mode had no effect on the duration of the prepupal stage in the present study might suggest that the effect found in earlier studies is due to the temperature (level and/or mode) experienced during larval development rather than the current temperature during the prepupal stage. In contrast to other developmental stages and to *O. lignaria*, the pupal stage seems to be not as sensitive to fluctuating vs. constant temperatures in *O. bicornis*, regardless of the temperature conditions experienced during larval development. Regarding the duration of complete development, the effect of T2-mode became not significant due to the lowered level of significance (see 3.3.4). Therefore, the lack of an effect in this study has to be treated with caution.

The significant effect of T1 on the duration of the prepupal phase supports the hypothesis that the temperature experienced during larval development might influence the duration of subsequent developmental stages: The bee offspring that were exposed to 17.5°C until cocoon spinning stayed significantly longer in the prepupal stage than the bee offspring at T1=10-25°C – independent of the actual ambient temperature during the prepupal stage (T2). One might argue that the overall longer duration of the prepupal stage in the bee offspring that were exposed to 17.5°C until cocoon completion might not have been a direct effect of temperature, but might have been caused by the potentially suboptimal conditions in this treatment (see above). We cannot completely exclude this possibility; anyway, the duration of the pupal stage was not affected by T1 anymore.

The most important result of this study was the effect of T2-level on the duration of the prepupal stage: Contrary to our expectation, the duration of the prepupal stage decreased with increasing temperature (Fig. 3.2). The same effect was found in the duration of the pupal stage and the duration of complete development. In ectotherms, development time usually decreases with increasing temperature due to the acceleration of biochemical processes at higher temperatures (Ratte 1984; Atkinson 1996). *Osmia* bees seem to follow this pattern regarding the duration of almost all developmental stages and complete development (Bosch and Kemp 2000; Radmacher and Strohm 2011a). However, the duration of the prepupal stage deviated from this general pattern in other studies: Kemp and Bosch (2005) reported a U-shaped thermal response of the duration of the prepupal stage for *O. lignaria* and Radmacher and Strohm (2011a) also detected an extension of the prepupal phase in the warm temperature treatments in *O. bicornis*. The extension of the prepupal period in warm

temperatures found by Kemp and Bosch (2005) in *O. lignaria* must have been induced by the actual temperature experienced in this phase since the bee offspring were not exposed to the experimental temperature treatments until cocoon completion in that study. We did the same in the present study for *O. bicornis*; however, we did not detect an extension of the prepupal phase. Therefore, we conclude that the extension of the prepupal phase in the warm treatments in *O. bicornis* observed by Radmacher and Strohm (2011a) was triggered by the temperature experienced during the preceding larval phase and not by the actual temperature during the prepupal stage. Thus, in contrast to Kemp and Bosch (2005), our results support the hypothesis that the duration of the prepupal stage in *O. bicornis* is influenced by the temperature experienced during previous stages. Effects of temperature experienced in early developmental stages on subsequent stages have hardly been investigated yet. However, such effects seem highly adaptive since in the study species, for example, the optimal adjustment of the duration of the prepupal stage might more strongly depend on the previous temperature and the resulting rate of development than on the actual temperature.

The regulation of development time plays an important role for ectotherm organisms in a seasonal environment. The life cycle has to be synchronized with local environmental conditions and adverse conditions (e.g. during winter in temperate regions) should be passed in a suitable stage (Gotthard 2001; Bale and Hayward 2010). Temperature usually affects the duration of development in insects (Ratte 1984). These effects are known to differ among different developmental stages, and some stages are more sensitive to temperature influences than others (Peterson et al. 2000; Rombough 2003; Folguera et al. 2010). If development is remarkably accelerated due to warm temperatures, multivoltine organisms might use the saved time for one or more additional generations in the growing season (Bale et al. 2002; Robinet and Roques 2010). In contrast, univoltine species might face the problem that they reach their overwintering stage (that is eventually particularly sensitive for higher temperatures) too early and might be exposed to unsuitably high temperatures.

The univoltine butterfly *Dasychira pudibunda* solved this problem by additional moults and larval stages when the larvae were exposed to high temperatures that resulted in a shorter duration of each larval instar (Geyspits and Zarankina 1963). For *Osmia* bees, it seems to be important to adjust adult eclosion to the onset of winter temperatures since long pre-wintering periods as adults led to elevated fat body depletion and decreased overwintering survival (Bosch et al. 2000, 2010; Bosch and Kemp 2004; Radmacher and Strohm 2011a).

Bosch et al. (2000) stated that *Osmia* bees from populations in warmer areas at lower latitudes remain in the prepupal stage for longer periods than bees that originate from more northerly populations. This is probably an adaptation of populations in lower latitudes with longer growing seasons and high temperatures to avoid long pre-wintering periods with their negative effects. The prepupal phase seems to be the developmental stage with the highest plasticity with regard to duration and with a lower susceptibility to warm temperatures than the adult stage (Bosch and Kemp 2000; Bosch et al. 2000, 2010; Sgolastra 2007). Our results suggest that the bee offspring might show phenotypic developmental plasticity by extending the prepupal phase to compensate for the acceleration of larval development due to warm temperatures. Since in the course of the current climate change overall mean temperatures are rising and the intensity and the frequency of heat waves in summer are increasing (IPCC 2007; EEA 2008), the offspring of *Osmia* bees might be increasingly confronted with warm temperatures during larval development. The described extension of the prepupal phase could be a suitable mechanism that allows the adjustment of adult eclosion under warmer conditions and that might represent a pre-adaptation that could help these important pollinators to cope with the consequences of climate change.

3.6 ACKNOWLEDGEMENTS

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CHAPTER 4

EFFECTS OF TEMPERATURE DURING DEVELOPMENT ON THE SYNAPTIC ORGANIZATION IN THE BRAIN OF THE SOLITARY BEE *OSMIA BICORNIS* (HYMENOPTERA, MEGACHILIDAE)

Arthropod Structure & Development, submitted

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4.1 SUMMARY

Development of insects might be affected by temperature. In the social honeybees, the temperature for their brood is effectively regulated. It has been shown that small deviations from the optimal rearing temperature affected synaptic brain organization, behaviour, and learning and memory of honeybee offspring. In contrast, the offspring of solitary bees are largely exposed to the temperature fluctuations of their environment. Therefore, solitary bees should be adapted to fluctuating temperatures during development and should show no or much smaller effects of developmental temperature on the brain than honeybees. To test this hypothesis, we reared both sexes of the solitary *Osmia bicornis* under three fluctuating and three constant temperature regimes for their entire development. We analysed neuropil size as well as density and number of microglomeruli (MG) in the mushroom body calyces and compared our results with data on honeybees. Although rearing temperature affected neuropil size, this effect was largely compensated by a reciprocal effect on MG density. As a result, overall MG numbers were hardly affected by developmental temperature in *O. bicornis*. Our results suggest that solitary bees are able to compensate for variable temperatures during development and, thus, are less susceptible to variations in rearing temperature than honeybees.

4.2 INTRODUCTION

The temperature experienced during development affects many important life history traits (e.g. development time and body size) and adult behaviour in insects, including bees (Ratte 1984; Atkinson 1994; Tautz et al. 2003; van Baaren et al. 2005; Becher et al. 2009; Radmacher and Strohm 2011a, b). Eusocial insects like termites, ants and honeybees actively control the microclimate in their nests and regulate temperature and humidity in a more or less constant range that is suitable for the development of their brood (Seeley and Heinrich 1981; Jones and Oldroyd 2006). Honeybees maintain brood nest temperatures in a remarkably narrow range of 33-36°C (Himmer 1927; Jones et al. 2004; Jones and Oldroyd 2006). Warmer or cooler temperatures had clear negative effects like elevated mortality and malformations (Himmer 1927). However, small deviations from the optimal temperature range of 34.5-35.5°C affected less obvious traits like behavioural performance (Tautz et al. 2003; Becher et al. 2009), short-term learning and memory (Jones et al. 2005), and the synaptic organization in the adult honeybee brain (Groh et al. 2004, 2006).

Unlike the eusocial honeybees, most bee species are solitary (Michener 2000). In solitary bees, there is no active regulation of the brood temperature. Thus, if solitary bees showed the same negative effects of deviations from the optimal temperatures during development as honeybees, they would suffer huge fitness losses due to the fluctuating weather conditions. Alternatively, developing solitary bees might show a broader reaction norm and compensate for temperature changes during their development. Here we tested these alternatives in the spring-active solitary Red mason bee *Osmia bicornis* (Linnaeus 1758, Hymenoptera, Megachilidae; formerly called *O. rufa*). *O. bicornis* is an abundant and important fruit tree pollinator in Central Europe (Westrich 1989; Krunić and Stanisavljević 2006a). The flight period of this polylectic species lasts from early April to mid-June. Females build their linear nests in a wide range of pre-existing cavities like holes in trees, soil or buildings. Females provision the brood cells with pollen from a diverse spectrum of plants and some nectar and lay one egg per cell. The larvae consume their provisions, spin a cocoon, go through a prepupal and a pupal stage, and eclose as adults in autumn inside their cocoons where they overwinter. Thus, the developing offspring of *O. bicornis* (and other cavity-nesting solitary bees) are exposed to the fluctuating ambient temperatures that are only slightly buffered by the material surrounding the nest.

For honeybees, Groh et al. (2004, 2006) have shown that the temperature during pupal development influences the number of distinct synaptic complexes, the microglomeruli (MG), in the calyces of the mushroom bodies (MBs) in the brains of adult bees. The MBs are prominent neuropils in the insect brain and represent multisensory integration centres that play an important role for learning, memory, and orientation (Menzel et al. 1994; Heisenberg 1998; Strausfeld et al. 1998, 2009; Zars 2000; Gronenberg 2001; Fahrbach 2006). The calyx of a honeybee MB comprises three subdivisions (lip, collar, and basal ring) that mainly receive olfactory (lip, basal ring) and visual (collar, basal ring) input from the antennal lobe and the medulla/lobula, respectively (Mobbs 1982; Gronenberg 2001). The nervous system of holometabolous insects develops during larval stages, but is completely remodelled during metamorphosis through apoptosis, neurogenesis, and synaptogenesis (Bauer 1904; Levine et al. 1995; Farris et al. 1999, 2004; Ganeshina et al. 2006). The temperature experienced during development might affect the physiological processes that underlie the formation of the adult brain and, thus, its final structure.

In this study, we investigated potential effects of rearing temperature on the synaptic organization (i.e. density and number of MG) in the brain of adult *O. bicornis*. We hypothesized that the offspring of *O. bicornis* should be adapted to fluctuating temperatures and expected that *O. bicornis* would show no or much smaller temperature effects on the MB calyces than honeybees. Therefore, we used a broader temperature range than used in the honeybees studies (Groh et al. 2004, 2006) and constant as well as fluctuating temperature treatments to increase the probability of detecting temperature effects. Moreover, the bee offspring were exposed to the experimental temperatures for their entire development (not only for the pupal stage as in Groh et al. (2004, 2006)). Since male bees are haploid they might be less able to compensate for environmental fluctuations (Lerner 1970; Clarke et al. 1992) and, thus, might be more likely to show temperature effects on the brain than females. Thus, we investigated the brains of both sexes to further increase the chance of detecting effects of different temperatures on brain structure. We visualized and quantified the MG in the *Osmia* calyces largely according to the protocol for honeybees and compared the results for *O. bicornis* and honeybee workers (Groh et al. 2004).

4.3 MATERIALS AND METHODS

4.3.1 Bees and temperature treatments

Bee offspring were obtained from a free-ranging *O. bicornis* population nesting in trap nests in an observation hut near the botanical garden of the University of Regensburg. The trap nests were made of dense styrofoam with transparent polycarbonate lids that allowed the continuous observation of nesting activity and the separation of recently completed brood cells from the nest (for more details see Strohm et al. 2002; Radmacher and Strohm 2011a). During the peak nesting season in 2008 (2 to 15 May), we separated brood cells with 1-3 day old eggs from their nests and brought them into the laboratory where they were sealed with paper and transparent foil. Then they were randomly allocated to one of seven temperature treatments: Three fluctuating regimes (10-25, 15-30, and 20-35°C) where temperature followed a sine curve and reached the eponymous minimum and maximum values once in 24 h, three constant temperatures that represented the mean temperatures of the fluctuating regimes (17.5, 22.5, and 27.5°C), and one control group (Hut) with natural temperature conditions. Ten to fourteen days after cocoon completion, individual brood cells were opened, the cocoon was cleaned of faeces, placed in a new trap nest, and put back in the according conditions. On 2 October 2008, all cocoons were transferred to a climate chamber with constant 4°C for wintering. For more details about the temperature treatments see Radmacher and Strohm (2011a).

4.3.2 Brain preparation and immunocytochemistry

Between 7 December 2008 and 28 January 2009, we used bees of each sex and temperature treatment from 49 different nests for the analysis of brain organization. Due to high mortality in the warm treatments (Radmacher and Strohm 2011a), we could not obtain similar sample sizes for all treatments (Males/Females: $N_{\text{Hut}}=7/6$, $N_{17.5^{\circ}\text{C}}=7/7$, $N_{22.5^{\circ}\text{C}}=7/7$, $N_{27.5^{\circ}\text{C}}=2/2$, $N_{10-25^{\circ}\text{C}}=7/7$, $N_{15-30^{\circ}\text{C}}=7/9$, $N_{20-35^{\circ}\text{C}}=9/3$). The brain preparations were largely made according to a protocol used for honeybees (Groh et al. 2004). The diapausing adult bees were taken out of their cocoons, anaesthetised with CO₂, and decapitated. The head capsule width (as a measure of body size) was determined to the nearest 0.1 mm under a stereo microscope with a measuring eyepiece. The brains were carefully dissected in cold physiological saline solution (130mM NaCl, 5mM KCl, 4mM MgCl₂, 5mM CaCl₂, 15mM Hepes, 25mM glucose, 160mM sucrose, pH 7.2), immediately immersed in cold 4% paraformaldehyde in 0.1M PBS (pH 7.2), and fixed overnight at 4°C. Based on initial experience, the fixation time was

reduced to 2-4 hours to reduce breakage of the brain tissue during cutting. After washing in PBS, the brains were embedded in a (6.25:1) mixture of ovalbumin (Sigma, A5253) and gelatine (Sigma, G2500) and postfixed in 4% paraformaldehyde in PBS overnight at 4°C. The brains were sectioned (thickness: 60µm) in a frontal plane using a vibratome (Leica VT 1000S, Nussloch, Germany).

The sections were washed in PBS (3x) and preincubated in 0.2% Triton X-100 (Merck, Germany) and 2% normal goat serum (NGS; Sigma, G9023) in PBS at room temperature for one hour. To label the neuronal F-actin, the sections were incubated in 0.5% Alexa Fluor® 488 phalloidin (Invitrogen, A12379), 1% NGS and 0.2% Triton X-100 in PBS for two days at 4°C in the dark. After washing in PBS (5x), the sections were mounted on glass slides in Vectashield® mounting medium (Vector Laboratories, H-1000) and stored at 4°C in the dark.

4.3.3 Imaging, estimating neuropil sizes, and counting MG

Brain sections were viewed with a confocal laser-scanning microscope (Zeiss LSM 510) equipped with an Ar-Laser (488nm). Pseudocolour images (resolution: 2048x2048 pixels) were taken of those sections that showed the four calyces cut transversely and where the pedunculi and the central body were clearly visible. The picture data were converted to TIFF-images using the Zeiss LSM Image Browser. Area estimations and MG counts were performed on the inner branch of the lateral calyx and the outer branch of the medial calyx on both sides (calyx branches 1-4 in Fig. 4.1A, B). The areas of the calyx branches (lip + collar, basal ring excluded) were estimated by marking the contours with the “magnetic lasso”-function in Adobe Photoshop Elements 2.0 (Fig. 4.1D). Unlike to the studies on honeybees (Groh et al. 2004, 2006), separate estimations for the areas of the lip and the collar or the loose and the dense area in the collar could not be reliably conducted since these areas showed no clear boundaries. Clearly identifiable MG were counted in three circles of 500 µm² each (generated with Inkscape, freeware, www.inkscape.org) per calyx branch (one in the lip, two in the collar; Fig. 4.1D). The counts were done blindly and independently by two persons. Differences between the two counts in each circle were 4.8 % on average. The numbers of MG per 500 µm² (called “MG density” in the following) were averaged for the lip (4 circles) and the collar (8 circles) for each brain. To obtain a measure for MG number that incorporates the size of the neuropil, the MG densities for the lip and the collar were separately extrapolated to the area of the whole calyx branch (lip + collar), resulting in an estimate for the total “MG number” of the lip or collar, respectively.

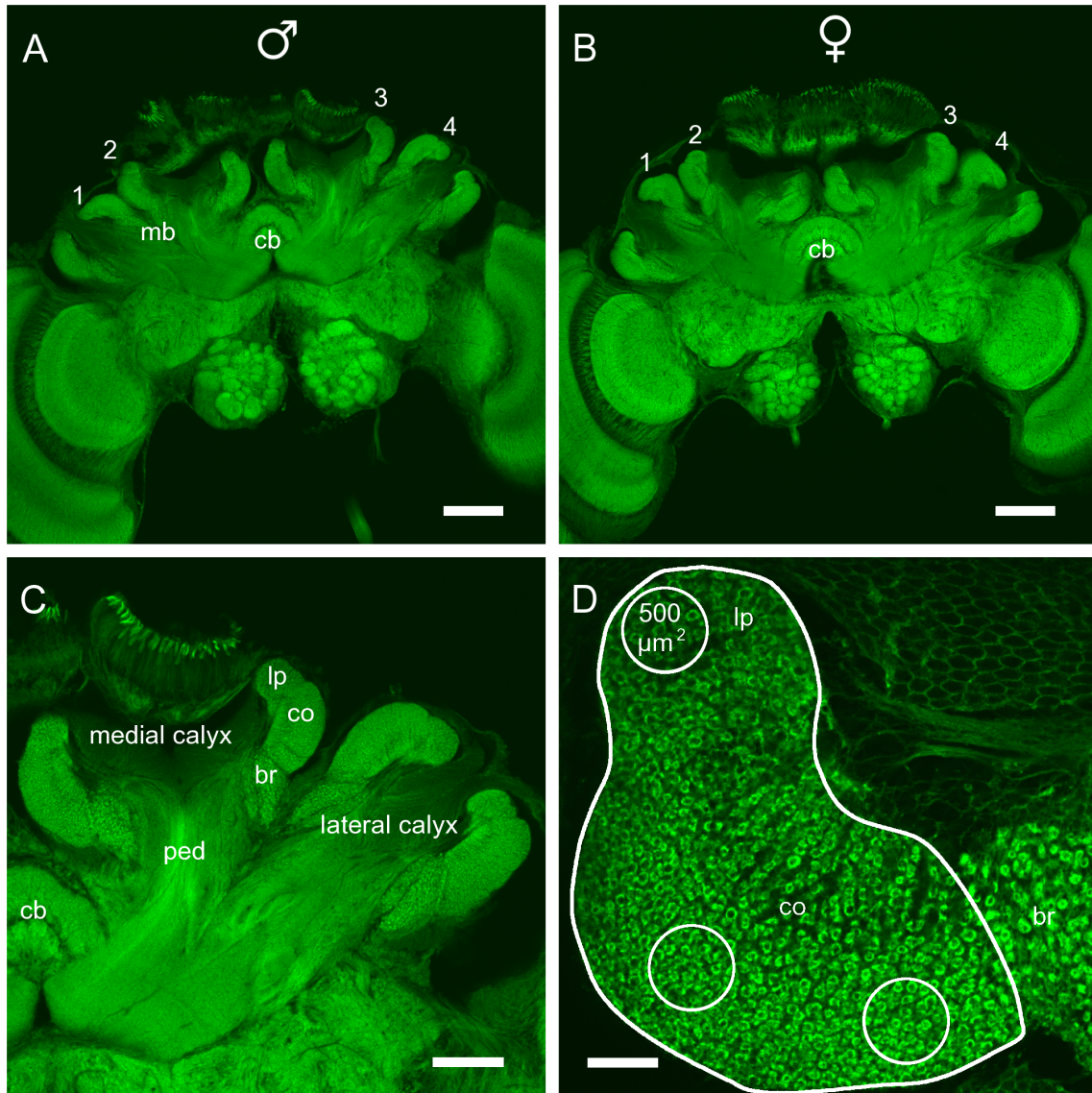


Figure 4.1: **A, B** Sections in a frontal plane of a male (**A**) and a female (**B**) *O. bicornis* brain, labelled with fluorophore-conjugated phalloidin. The mushroom bodies (mb) and the central body (cb) are clearly visible. Calyx branches used for area estimations and MG counts are indicated by numbers (1-4); scale bars = 200 μm . **C** Mushroom bodies with their subdivisions lip (lp), collar (co), basal ring (br) and peduncule (ped); scale bar = 100 μm . **D** Calyx branch with MG. The measured calyx branch area (lip + collar) and three circles of 500 μm^2 in which MG were counted are indicated; scale bar = 20 μm .

4.3.4 Data analyses

All statistical analyses were performed using PASW Statistics 18 (i.e. SPSS 18.0). To investigate potential effects of temperature and sex on head capsule width, an ANOVA with temperature level (cool, medium, and warm), temperature mode (constant vs. fluctuating), and sex as fixed factors was conducted. Separate ANOVAs for males and females with

temperature level and temperature mode as fixed factors were conducted to investigate temperature effects on the calyx branch area, the MG density (lip/collar), and the MG number (lip/collar). The control group (Hut) was not included in the analyses, but is presented in the figures as a reference for the respective variable under natural conditions. To test for a general relationship between the calyx branch area and the MG density (lip/collar), correlation analyses (Pearson) including all bees (irrespective of sex or temperature treatment) were conducted.

In case of a non significant result for the effect of temperature level, we used power analysis (G-Power 3.1.2 freeware; Erdfelder et al. 1996) to estimate the type II error “ β ”. This analysis calculates the probability that we would have detected an effect in *O. bicornis* if it was as large as in honeybees. The respective effect sizes for honeybees were determined based on the studies by Groh et al. (2004; derived from the values for the differences between 29 and 34.5°C in figure 2).

To compare the magnitude of the temperature influences on the calyces that we found in *O. bicornis* with those reported for honeybees (Groh et al. 2004), we determined the largest difference that was found between two (mean) values for calyx branch area, MG density (lip/collar), and MG number (lip/collar) for a temperature difference of about 5°C. The higher value was defined as 100% and the difference (in %) to the lower value was calculated. This difference is further referred to as “relative temperature effect”. The data for honeybees were derived from figure 2 of Groh et al. 2004 (differences between 29.5 and 34.4°C, except for lip MG density (between 30 and 34.5°C)). The data for *O. bicornis* were analysed separately for constant and fluctuating temperature conditions.

4.4 RESULTS

4.4.1 Immunofluorescence labelling of neuropils

The fluorophore-conjugated phalloidin labelled all synaptic neuropils in the brain of *O. bicornis* due to its affinity to dendritic F-actin (Wieland 1987; Rössler et al. 2002; Frambach et al. 2004; Fig. 4.1A, B). The calyces of the MBs were intensely labelled and small circular structures were clearly visible (Fig. 4.1C, D). These circular structures represent the microglomeruli (MG), i.e. distinct synaptic complexes that consist of dendritic spines mostly

from Kenyon cells that surround a central, non-labelled bouton that is formed by axons from projection neurons (Gronenberg 2001; Frambach et al. 2004; Groh et al. 2004, 2006). The overall shape of the neuropils in the brain of *O. bicornis* was similar to that of honeybees (Groh et al. 2004, 2006). Except for a general difference in brain size between the sexes, we noticed no conspicuous differences in gross morphology of the brain or of the MB between the sexes or temperature treatments.

4.4.2 Body size and calyx branch size

Head capsule width was significantly affected by sex ($F=61.26$, $df=1$, $p<0.001$), temperature level ($F=13.933$, $df=2$, $p<0.001$), and temperature mode ($F=5.892$, $df=1$, $p=0.019$). Females were larger than males and body size tended to decrease with increasing temperatures and to be smaller in constant (vs. fluctuating) temperatures (Fig. 4.2A). In both sexes, the calyx branch area was significantly affected by temperature level (Males/Females: $F=4.044/7.736$, $df=2/2$, $p=0.027/0.002$) and temperature mode (Males/Females: $F=6.195/4.972$, $df=1/1$, $p=0.018/0.034$) with an apparent maximum at medium temperatures and larger calyx branches at fluctuating (vs. constant) temperatures (Fig. 4.2B).

4.4.3 MG density and MG number

Temperature level (TL) significantly affected MG density in the lip of both sexes, but not temperature mode (TM) (TL Males/Females: $F=10.255/14.285$, $df=2/2$, $p<0.001/<0.001$; TM Males/Females: $F=1.73/2.571$, $df=1/1$, $p=0.197/0.12$). In the collar, we detected significant effects of temperature level and temperature mode in both sexes (TL Males/Females: $F=10.986/5.814$, $df=2/2$, $p<0.001/=0.008$; TM Males/Females: $F=7.242/5.137$, $df=1/1$, $p=0.011/0.031$). The MG densities in both calyx subdivisions were elevated at warm temperatures, in particular at 27.5°C (Fig. 4.2C, D). In general, the MG densities significantly decreased with increasing calyx branch area (lip: $r=-0.480$, $N=87$, $p<0.001$; collar: $r=-0.649$, $N=87$, $p<0.001$; Fig. 4.3).

We detected no significant effect of temperature level on estimated total MG number in the lip in both sexes (Males/Females: $F=2.725/2.623$, $df=2/2$, $p=0.08/0.09$). Temperature mode significantly affected the lip MG number in females ($F=5.057$, $df=1$, $p=0.032$) with higher MG number in fluctuating (vs. constant) temperatures, but not in males ($F=2.231$, $df=1$, $p=0.145$; Fig. 4.2E). In the collar (Fig. 4.2F), there were no temperature effects on MG number in males (TL: $F=2.142$, $df=2$, $p=0.134$; TM: $F=1.549$, $df=1$, $p=0.222$). In

females, collar MG number was affected by temperature level ($F=5.65$, $df=2$, $p=0.008$) with a maximum at medium temperatures, but not by temperature mode ($F=2.036$, $df=1$, $p=0.164$; Fig 4.2F).

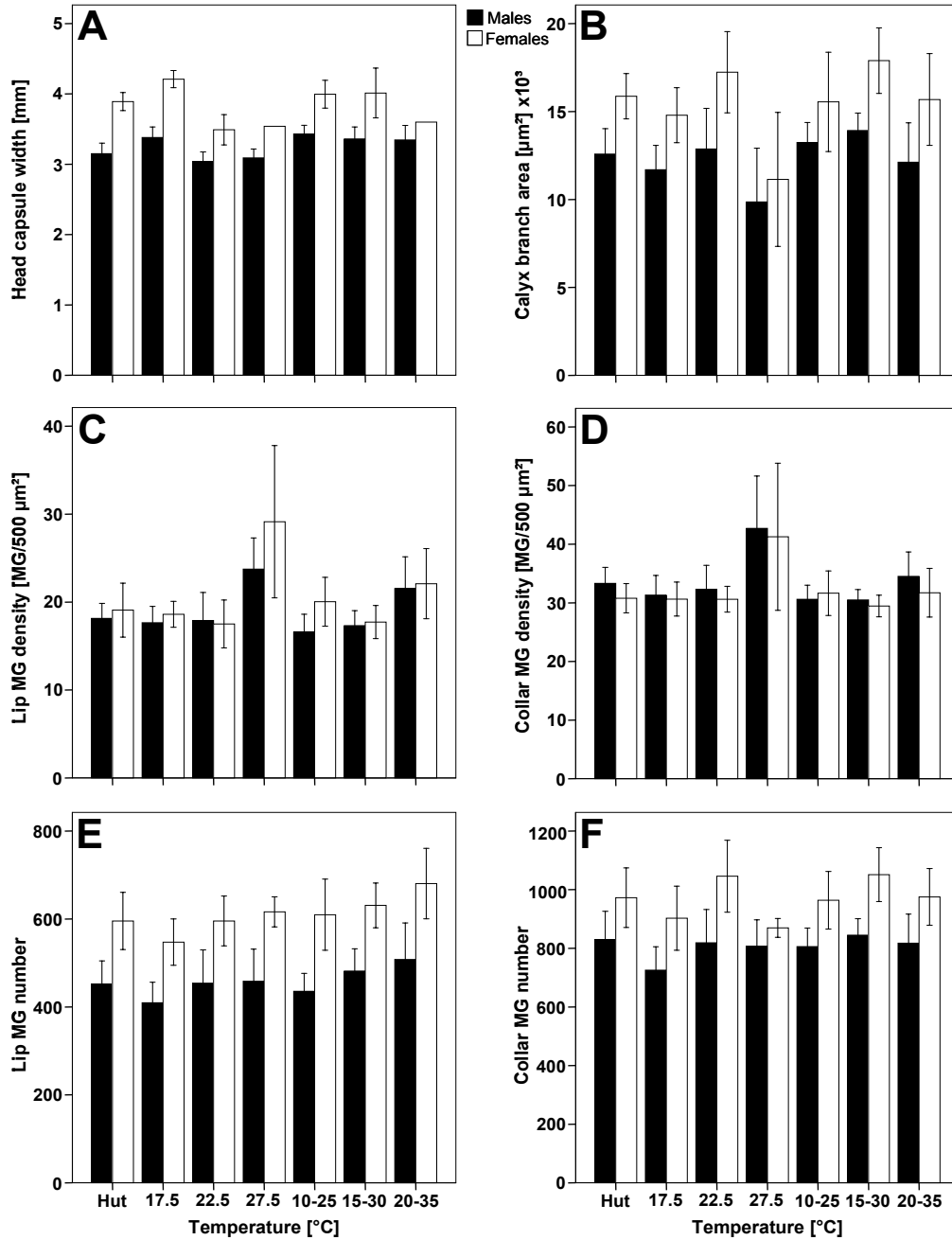


Figure 4.2: Temperature effects on head capsule width (A), calyx branch area (B), MG density in the lip (C) and the collar (D), and MG number (i.e. extrapolated density to calyx branch area) in the lip (E) and the collar (F) in *O. bicornis*. The bars (black for males, white for females) represent the mean \pm SD. Three fluctuating temperature regimes (10-25, 15-30, and 20-35°C), their constant mean temperatures (17.5, 22.5, and 27.5°C), and one control with natural temperature conditions (Hut) were investigated. Sample sizes for A: Males/Females: $N_{\text{Hut}}=6/6$, $N_{17.5^\circ\text{C}}=6/6$, $N_{22.5^\circ\text{C}}=6/6$, $N_{27.5^\circ\text{C}}=2/1$, $N_{10-25^\circ\text{C}}=6/5$, $N_{15-30^\circ\text{C}}=6/8$, $N_{20-35^\circ\text{C}}=8/1$. Sample sizes for B-F: Males/Females: $N_{\text{Hut}}=7/6$, $N_{17.5^\circ\text{C}}=7/7$, $N_{22.5^\circ\text{C}}=7/7$, $N_{27.5^\circ\text{C}}=2/2$, $N_{10-25^\circ\text{C}}=7/7$, $N_{15-30^\circ\text{C}}=7/9$, $N_{20-35^\circ\text{C}}=9/3$.

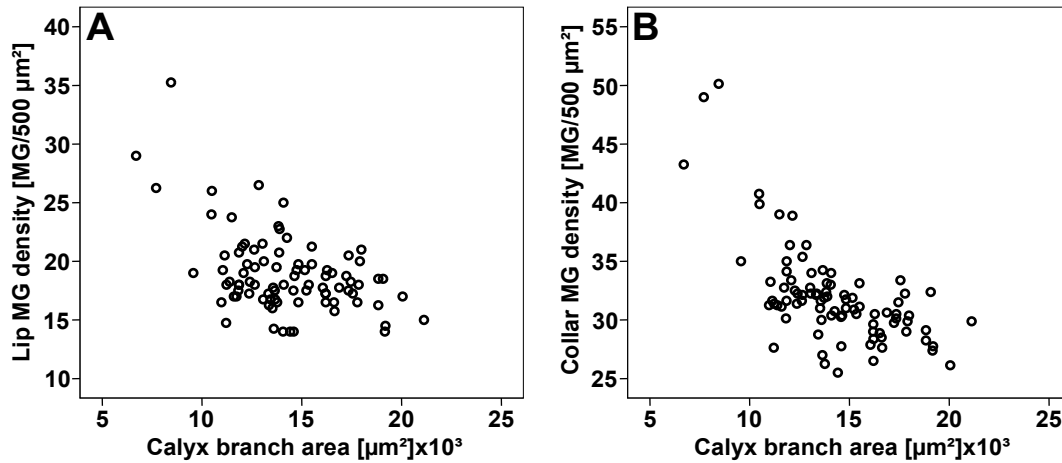


Figure 4.3: Association between the calyx branch area and the MG density in the lip (A) and in the collar (B) for all investigated bees (irrespective of sex or temperature treatment; N=87).

Since we found non significant effects of temperature level on the MG number in the lip (males and females) and in the collar (males), we conducted power analyses to assess the probability of false negative results. The estimated probability ($1-\beta$; power) of detecting temperature effects similar to those in honeybees (Groh et al. 2004) with our data would have been >0.999 in all tested cases.

4.4.4 Comparison of relative temperature effects on honeybees and *O. bicornis*

The relative temperature effects on calyx branch area, MG density in the lip and collar, and MG number in the lip and collar of honeybees and *O. bicornis* are listed in Table 4.1. In general, the relative temperature effect on calyx branch area was smaller in *O. bicornis* than in honeybees, particularly under fluctuating conditions. For MG density in the lip, the relative temperature effects in honeybees and *O. bicornis* were rather similar, except for *Osmia* females in constant temperatures where the detected relative temperature effect was relatively high. The relative temperature effect on MG density in the collar was similar in honeybees and *O. bicornis* in constant temperatures, but this effect was smaller in *Osmia* bees that were exposed to fluctuating temperatures. We found remarkable differences between honeybees and *O. bicornis* in the magnitude of temperature effects on the MG number in the lip and the collar: Whereas the difference between the highest and the lowest value of these variables were about 50% in honeybees, the relative temperature effect did not exceed 17% in *O. bicornis*. Mostly, it was lower than 10%, in particular under fluctuating conditions.

Table 4.1: Comparison of the relative temperature effects on honeybee workers (*Apis*, data derived from Fig. 2 in Groh et al. 2004) and *O. bicornis* (*Osmia*). Listed are the largest differences (in %, see below) that were found between two mean values of each variable (i.e. calyx branch area, MG density in lip and collar, and MG number in lip and collar) for a temperature difference of about 5°C (4.5-5.5°C for honeybees). The higher value was defined as 100% and the difference to the lower value was calculated as the difference in % and defined as “relative temperature effect”. The data for *O. bicornis* were analysed separately for males and females and for constant and fluctuating temperatures (T).

Variable	<i>Apis</i>	<i>Osmia</i> ♂ constant T	<i>Osmia</i> ♀ constant T	<i>Osmia</i> ♂ fluctuating T	<i>Osmia</i> ♀ fluctuating T
Calyx branch area	43	24	36	13	13
Lip MG density	24	17	40	20	20
Collar MG density	24	24	21	12	7
Lip MG number	47	1	3	5	7
Collar MG number	59	11	17	5	8

4.5 DISCUSSION

4.5.1 Labelling of neuropils

As in honeybees and other insects (e.g. Frambach et al. 2004; Groh et al. 2004), the fluorophore-conjugated phalloidin labelling of the synaptic neuropils including the circular postsynaptic parts of the MG (i.e. the dendritic spines from Kenyon cells) was successful in *O. bicornis*. Unfortunately, in preliminary studies we had no success in staining the presynaptic part of the MG (i.e. the central bouton formed by axons from projection neurons) by anti-synapsin labelling as it has been done repeatedly in studies on MG (Frambach et al. 2004; Groh et al. 2004, 2006; Krofczik et al. 2008; Hourcade et al. 2010; Stieb et al. 2010). However, the phalloidin-labelled circular structures (spherical, ellipsoidal or irregular in shape) are characteristic for MG (Yasuyama et al. 2002; Frambach et al. 2004; Leiss et al. 2009) and were clearly visible and countable (Fig. 4.1D). Since we determined MG densities and numbers in the same way for all temperature treatments and since we did not compare absolute values but relative temperature effects in % in *O. bicornis* and honeybees, it is unlikely that the single labelling has biased our data.

4.5.2 Body size and neuropil size

As expected based on the sexual size dimorphism in *O. bicornis* (Maddocks and Paulus 1987), females were larger than males (represented by head width). Since larger bees usually have larger brains (Mares et al. 2005; Withers et al. 2008), it is not surprising that females had larger calyces than males, irrespective of temperature treatment. However, regarding the temperature effects, head capsule width and calyx branch area showed different patterns (Fig. 4.2A, B). According to the temperature-size rule and previous studies on this species (Atkinson 1994; Radmacher and Strohm 2010, 2011a), body size decreased with increasing temperature, though this effect was not as evident as in our previous studies (possibly due to the comparatively small sample of bees for brain preparations). In contrast, the calyx branch area was maximal at medium temperatures under constant and fluctuating conditions. These different patterns suggest that the calyx branch area was directly affected by rearing temperature and not only indirectly by temperature effects on body size. In contrast to changes in calyx size due to age, experience or social interactions that have been reported for ants (Gronenberg et al. 1996), wasps (O'Donnell et al. 2004; Molina and O'Donnell 2007, 2008), honeybees (Withers et al. 1993; Durst et al. 1994; Fahrbach et al. 1997; Groh et al. 2006; Maleszka et al. 2009; Stieb et al. 2010), bumble bees (Riveros and Gronenberg 2010), sweat bees (Smith et al. 2010), and *O. lignaria* (Withers et al. 2008), the effect of developmental temperature on the size and structure of the insect brain received less attention. Wang et al. (2007) found a decreased calyx size in *Drosophila* when the larvae and pupae were exposed to ecologically-relevant heat stress during development. For honeybees, Groh et al. (2004) reported that particularly the lip area was affected by temperature.

4.5.3 MG density and MG number

The significant temperature effects on the MG density in the lip and the collar that we detected for male and female *O. bicornis* were probably mainly due to the elevated MG densities in the warm treatments, particularly in constant 27.5°C (Fig. 4.2C, D). Thus, the MG densities were highest in the temperature treatment that resulted in the lowest calyx size (Fig. 4.2B). This result suggests that the temperature effects on calyx size might have been compensated by the increased MG density in the warm treatments, keeping the overall number of MG more or less constant. The significant negative correlation between calyx size and MG density in the lip and the collar supports the hypothesis that (temperature-induced) differences in neuropil size were counterbalanced by MG density, resulting in comparable MG numbers in individuals of different (calyx) size.

The compensation of calyx size differences by MG density seems to have been largely successful since we found no significant effects of temperature level on the total MG number (i.e. the number that resulted from the extrapolation of MG density of the lip and the collar to calyx branch area), except for the collar MG number in females that was maximal at medium temperatures – like the calyx branch area. However, the temperature effect on the collar MG number in females was remarkably smaller than that on the calyx branch area, particularly under constant temperature conditions (Tab. 4.1), indicating that temperature influence on the calyx size was at least attenuated by counterbalancing MG density. The power analyses showed that temperature effects similar to those in honeybees would have been detected with a very high probability and that the non significant effects on MG number in *O. bicornis* were not due to inadequate sample sizes or high variance. There were no indications for a lower capability of the haploid *O. bicornis* males (vs. the diploid females) to compensate for environmental fluctuations during development as it has been reported for honeybees (Clarke et al. 1992).

Recently, several studies revealed that the density, number, and/or size of MG (or of parts of it, respectively) is influenced by caste, age, and experience in ants and honeybees (Seid et al. 2005; Groh et al. 2006; Groh and Rössler 2008; Krofczik et al. 2008; Muenz et al. 2008; Stieb et al. 2010). In addition, MG size increased due to olfactory/visual associative training in cockroaches (Lent et al. 2007) and the formation of olfactory long-term memory increased the lip MG density in honeybees whereas the lip size remained constant (Hourcade et al. 2010). Thus, the synaptic organization in the MB calyx of insects shows a remarkable plasticity. This plasticity was also evident regarding the effects of developmental temperature on neuropil size, MG density and MG number in honeybees, particularly in the lip of their mushroom bodies (Groh et al. 2004, 2006). Our study revealed such plasticity in response to developmental temperature for neuropil size and MG density, but considerable developmental stability for the overall MG number in *O. bicornis*.

4.5.4 Comparison of temperature effects on honeybees and *O. bicornis*

Since the offspring of solitary bees are naturally exposed to fluctuating and sometimes extreme ambient temperatures, we expected that *O. bicornis* would show no or much smaller temperature effects on the MB calyces than honeybees whose offspring is probably adapted to more or less constant rearing temperatures. We found that rearing temperature affected the neuropil size in *O. bicornis*, but this effect was smaller than in honeybees (particularly

under fluctuating conditions), supporting our hypothesis. At the first glance, our results regarding MG density seem not to be in line with our hypothesis since the sizes of the temperature effect were similar or even higher in *O. bicornis* compared to honeybees (Tab. 4.1). However, the pattern of the temperature effect on MG density was different in the two bee species: In *O. bicornis*, the effect on MG density counterbalanced the temperature effect on neuropil size since MG density increased when the neuropil size decreased. In honeybees, the temperature effects on neuropil size and MG density were rather additive since both variables were maximal at the medium optimal temperature of 34.5°C, declining in lower and higher temperatures (Groh et al. 2004, Fig. 2). Thus, the combination of the effects on neuropil size and MG density resulted in a strong effect on overall MG number in honeybees whereas there were no or only small detectable effects on MG number in *O. bicornis*. Thus, the overall MG numbers are much less influenced by temperature in *O. bicornis* than in honeybees.

Although we included males in our investigation and used constant as well as fluctuating temperature regimes within a broad temperature range for the entire bee development, we detected no or only small temperature effects on the synaptic organization (particularly MG number) in the MBs of *O. bicornis*, probably due to a counterbalancing relationship between neuropil size and MG density in this bee species. Therefore, our results support the hypothesis that solitary bees are able to compensate for fluctuating and sometimes non-optimal temperatures during development and, thus, are less susceptible to variations in temperature during development than honeybees.

4.6 ACKNOWLEDGEMENTS

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CHAPTER 5

DOES TEMPERATURE EXPERIENCED DURING METAMORPHOSIS AFFECT THE SYNAPTIC ORGANIZATION IN THE BRAIN OF A SOLITARY BEE?

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5.1 SUMMARY

Developmental temperature can affect important life history traits in insects. The social honeybees keep the temperature for their brood rather constant. Small deviations from the optimal temperature during pupal development affected synaptic organization in the brain, behaviour, and cognitive abilities of adults. Recently, we found that in the solitary bee *Osmia bicornis* the synaptic brain organization was much less affected by rearing temperature than in honeybees when offspring was exposed to three fluctuating and three constant temperature regimes for their entire development. Here we tested whether *O. bicornis* would also be less affected than honeybees when bee offspring experienced the experimental temperatures only during post-larval development, excluding potential acclimation to the respective temperatures during larval development. We analysed neuropil size as well as density and number of microglomeruli in the mushroom body calyces and compared our results with data on honeybees and our previous study on *O. bicornis*. Though we found indications for some acclimation to the experimental temperatures during larval development, the brains of *O. bicornis* were much less affected by pupal rearing temperature compared to honeybees. This supports our hypothesis that the offspring of solitary bees should be adapted and, thus, less susceptible to fluctuating temperatures during development.

5.2 INTRODUCTION

For ectotherms, temperature is one of the most important environmental factors and the temperature experienced during development can affect important life history traits (e.g. development time, body size, and fecundity) and adult behaviour (Ratte 1984; Atkinson 1994; Scott et al. 1997; Tautz et al. 2003; Stillwell and Fox 2005; van Baaren et al. 2005; Bonte et al. 2008; Becher et al. 2009; Radmacher and Strohm 2011a). The eusocial honeybees actively control the microclimate in their nests, providing a more or less constant and suitable rearing temperature for their brood (33-36°C, mainly 34.5-35.5°C; Seeley and Heinrich 1981; Jones et al. 2004; Jones and Oldroyd 2006). Deviations from this narrow temperature range have been shown to affect morphology, mortality and susceptibility to pesticides (Himmer 1927; Medrzycki et al. 2010), behavioural performance (Tautz et al. 2003; Becher et al. 2009), short-term learning and memory (Jones et al. 2005) as well as the synaptic organization in the brain of adult honeybees (Groh et al. 2004, 2006). In contrast to honeybee colonies, solitary bees can not regulate the temperature for their brood. Their offspring is more or less exposed to fluctuating ambient temperatures, only slightly buffered by nesting material (particularly in cavity-nesting species). Therefore, solitary bees should have a greater capability to compensate for temperature fluctuations and occasionally non-optimal temperatures during development than honeybees.

In the studies on honeybees, Groh et al. (2004, 2006) investigated the effects of rearing temperature on neuropil size and the density and number of distinct synaptic complexes, the microglomeruli (MG), in the calyces of the mushroom bodies (MBs). The MBs are known as multisensory integration centres that play an important role for learning, memory, and orientation in the insect brain (Menzel et al. 1994; Heisenberg 1998; Strausfeld et al. 1998, 2009; Zars 2000; Gronenberg 2001; Fahrbach 2006). In the honeybee MB, the calyx comprises three subdivisions - lip, collar, and basal ring - that differ in their main sensory input (lip: olfactory, collar: visual, basal ring: both; Mobbs 1982, Gronenberg 2001). In holometabolous insects, the final structure of the adult nervous system results from a process of complete remodelling (via apoptosis, neurogenesis and synaptogenesis) that occurs during metamorphosis (Bauer 1904; Levine et al. 1995; Farris et al. 1999, 2004; Ganeshina et al. 2006). The prevailing temperature during metamorphosis might affect the physiological processes that underlie the remodelling and, thus, the final brain structure.

In these studies on temperature effects on the honeybee brain (Groh et al. 2004, 2006), the experimental temperatures were applied only during the pupal stage during which metamorphosis takes place. Recently, we investigated the effects of different constant and fluctuating rearing temperatures on the synaptic organization in the adult brain of the solitary bee *Osmia bicornis* (Linnaeus 1758, Hymenoptera, Megachilidae; formerly called *O. rufa*) to test the hypothesis that solitary bees, unlike honeybees, are able to compensate for the negative effects of non-optimal developmental temperatures (Radmacher and Strohm 2011c). In this study on *O. bicornis*, bee offspring were exposed to the experimental temperatures during their entire development (in contrast to the honeybee studies) to increase the probability of detecting significant temperature effects on the brain. According to our hypothesis that solitary bees should be better adapted to different temperatures, we found no or considerably smaller temperature effects on the MG number compared to honeybees (Groh et al. 2004). This was mainly due to reciprocal effects of temperature on neuropil size on the one hand and MG density on the other hand with the result that total MG number was only slightly affected (Radmacher and Strohm 2011c). However, it might be argued that the bee offspring might have somehow acclimatized to the respective temperature during larval development, resulting in a buffering of temperature effects during metamorphosis. To test for a potential influence of such an acclimatization effect of the temperature during larval development and to provide a better comparability with the study on honeybees (Groh et al. 2004), in the present study we exposed *O. bicornis* offspring to the respective experimental temperatures only for the post-larval development (i.e. the prepupal and pupal stage). We still hypothesized that *O. bicornis* would show no or much smaller temperature effects on brain organization than honeybees, even though there was no possibility for an acclimatization to the respective temperatures during larval development.

5.3 MATERIALS AND METHODS

5.3.1 Bees and temperature treatments

O. bicornis is an abundant solitary bee and an important fruit tree pollinator in Central Europe (Westrich 1989; Krunić and Stanisavljević 2006a). The flight period of this univoltine and polylectic species lasts from early April to mid-June. Females build their linear nests in pre-existing cavities, provision the brood cells with pollen and some nectar and lay one egg per brood cell. The offspring develop during summer and eclose as adults in autumn inside their

cocoons where they overwinter.

Bee offspring were obtained from a free-ranging population nesting in trap nests in an observation hut near the botanical garden of the University of Regensburg. The trap nests were made of dense styrofoam with transparent polycarbonate lids and allowed manipulation of the brood cells (for more details about the trap nests see Strohm et al. 2002; Radmacher and Strohm 2011a). During the peak nesting season in 2009 (14 to 26 April), we separated recently completed brood cells with 1-3 day old eggs from the nests and brought them into the laboratory where they were sealed with paper and transparent foil. Brood cells were randomly allocated to either a constant (17.5°C) or a fluctuating (10-25°C) temperature treatment for the development from egg until cocoon completion (called “T1” in the following for “temperature treatment 1”). In a previous study, these conditions were appropriate for bee development (Radmacher and Strohm 2011a). As in the preceding study (Radmacher and Strohm 2011c), we investigated both sexes in order to increase the probability to detect any effects.

On the day of cocoon completion (i.e. when the cocoon was completely darkened), the individual brood cells were opened, the cocoons were cleaned of faeces and placed in new trap nests, randomly allocated to one of seven temperature treatments for the subsequent development inside the cocoon (called “T2” in the following). The temperature treatments were: Three fluctuating temperature regimes (10-25, 15-30, and 20-35°C) in which temperature followed a sine curve and reached the eponymous minimum and maximum values once in 24 h, three constant temperatures that represented the mean temperatures of the fluctuating temperature regimes (17.5, 22.5, and 27.5°C) and one control group (“Hut”) that was exposed to the natural temperature conditions. These were the same seven temperature treatments as were used for the entire bee development in previous studies (Radmacher and Strohm 2011a, c). For more details about the temperature treatments see Radmacher and Strohm (2011a).

After cocoon completion, bees undergo a prepupal and a pupal stage and eclose as adults inside their cocoons. Then they experience a pre-winter phase until the temperature drops and bees enter the wintering phase. The duration of all developmental phases until adult eclosion is strongly affected by temperature and usually decreases with increasing temperature (Radmacher and Strohm 2011a, b). In an earlier study, mortality of adult bees

was considerably increased in warm treatments, probably due to a long pre-wintering period in high temperatures (Radmacher and Strohm 2011a). As a consequence, the sample sizes for brain preparations in the warm treatments were reduced (Radmacher and Strohm 2011c). Therefore, in this study we adjusted the duration of the pre-winter phase with regard to the duration of development. Since the pre-winter duration corresponds to nearly one third of the duration of development from egg to adult under natural conditions (data of the control group in Radmacher and Strohm 2011a), we specified one third of the estimated mean duration of development for each temperature group (T1 and T2; based on daily monitoring of bee development, Radmacher and Strohm 2011b) as the pre-wintering period. After the pre-wintering period in the respective T2, each group was individually transferred to constant 17.5°C for one week and then to constant 4°C for wintering.

5.3.2 Brain preparations and immunocytochemistry

We used eight males and eight females of each T2-treatment from a total of 45 different nests for the analysis of brain organization. About one half of each sample had experienced a T1 of 17.5°C or 10-25°C, respectively. Brain preparations were made between 30 October and 25 November 2009, largely according to a protocol that was used for honeybees (Groh et al. 2004) and in our previous study on *O. bicornis* (Radmacher and Strohm 2011c). The diapausing bees were removed from their cocoons, anaesthetised with CO₂ and decapitated. Head capsule width was determined to the nearest 0.1 mm with a measuring eyepiece. Brains were dissected in cold physiological saline solution (130mM NaCl, 5mM KCl, 4mM MgCl₂, 5mM CaCl₂, 15mM Hepes, 25mM glucose, 160mM sucrose, pH 7.2), immediately immersed in cold 4% paraformaldehyde in 0.1M PBS (pH 7.2), fixed for 2-4 hours at 4°C, and washed in PBS. After embedding in a mixture of ovalbumin and gelatine (6.25:1) and postfixing in 4% paraformaldehyde in PBS overnight at 4°C, brains were sectioned (60µm) in a frontal plane with a vibratome (Leica VT 1000S, Nussloch, Germany). The sections were washed in PBS (3x) and preincubated in 0.2% Triton X-1000 and 2% normal goat serum (NGS) in PBS for one hour at room temperature. To label the dendritic F-actin, the sections were incubated in 0.5% Alexa Fluor® 488 phalloidin (Invitrogen, A12379), 1% NGS and 0.2% Triton X-100 in PBS for two days at 4°C in the dark. Then they were washed in PBS (5x) and mounted on glass slides in Vectashield® mounting medium (Vector Labs, H-1000).

5.3.3 Imaging, estimating neuropil sizes, and counting MG

The methods for imaging and data evaluation were the same as in our previous study

(Radmacher and Strohm 2011c). Brain sections were viewed with a confocal laser-scanning microscope (Zeiss LSM 510) and pseudocolour images were taken of those sections that showed the four calyces cut transversely and where the pedunculi and the central body were clearly visible (Fig. 5.1A). Estimations of the calyx branch area (lip + collar, basal ring excluded) using the “magnetic lasso”-function of Adobe Photoshop Elements 2.0 and MG counts were performed on the inner branch of the lateral calyx and the outer branch of the medial calyx on both sides of the brain (calyx branches 1-4, Fig. 5.1A, B). MG were counted blindly and independently by two persons in three circles of $500\ \mu\text{m}^2$ each per calyx branch (one in the lip, two in the collar; Fig 5.1C). Differences between the two counts in each circle were 8.8 % on average. For each brain, the numbers of MG per $500\ \mu\text{m}^2$ (called “MG density” in the following) were separately averaged for the lip (values from 4 circles) and the collar (values from 8 circles). To obtain a measure for MG number that incorporates the size of the neuropil, the MG densities for the lip and the collar were separately extrapolated to the area of the whole calyx branch (lip + collar), resulting in “MG number” of the lip or collar, respectively.

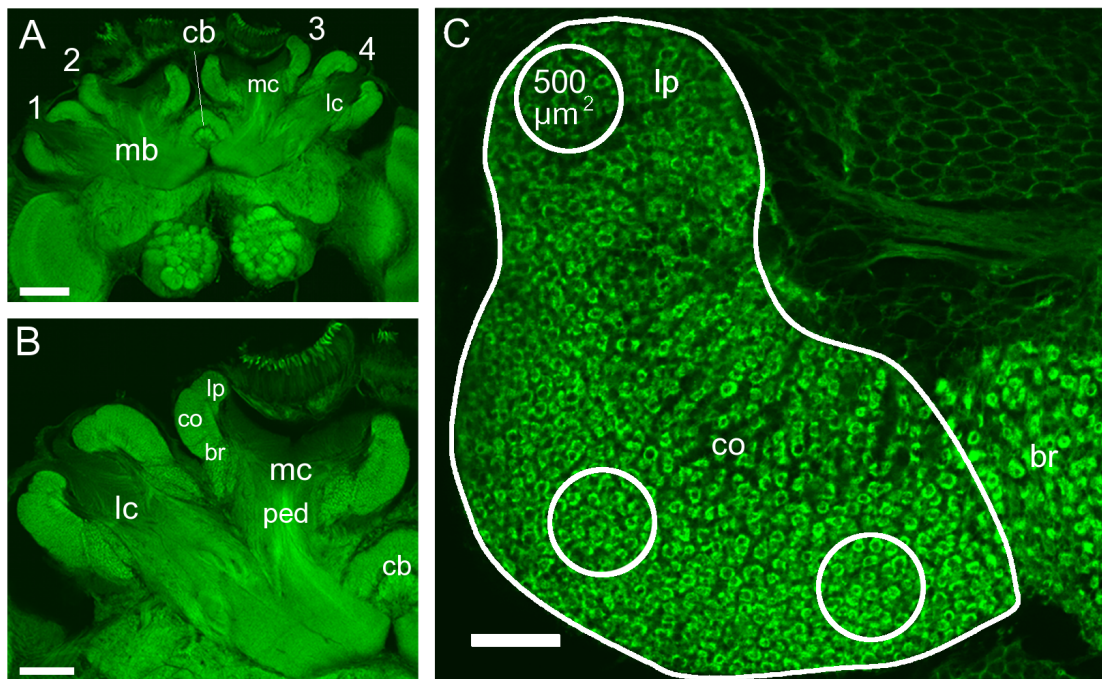


Figure 5.1: **A** Section in a frontal plane of a male *O. bicornis* brain, labelled with fluorophore-conjugated phalloidin. The mushroom bodies (mb) and the central body (cb) are clearly visible. Calyx branches used for area estimations and MG counts are indicated by numbers (1-4); scale bar = $200\ \mu\text{m}$. **B** Mushroom bodies with the lateral (lc) and medial (mc) calyx, the peduncule (ped), and the three calyx branch subdivisions lip (lp), collar (co), and basal ring (br); scale bar = $100\ \mu\text{m}$. **C** Calyx branch with MG. The measured calyx branch area (lip + collar) and the three circles of $500\ \mu\text{m}^2$ in which MG were counted are indicated; scale bar = $20\ \mu\text{m}$.

In most studies on MG in honeybees and other insects, a double labelling with fluorophore-conjugated phalloidin (staining of the dendritic spines mostly from Kenyon cells) and anti-synapsin (staining of the central bouton, formed by axons from projection neurons) was conducted (Frambach et al. 2004; Groh et al. 2004, 2006; Krofczik et al. 2008; Hourcade et al. 2010; Stieb et al. 2010). However, as in our previous study on *O. bicornis* (Radmacher and Strohm 2011c), we used only the fluorophore-conjugated phalloidin labelling since we had no success with anti-synapsin labelling in preliminary studies. However, the phalloidin-labelled circular structures are rather characteristic for MG (Yasuyama et al. 2002; Frambach et al. 2004; Leiss et al. 2009) and since we determined MG densities and numbers in the same way for all temperature treatments and did not compare absolute values but relative temperature effects (in %, see below), it is unlikely that the single labelling caused biases in our data.

5.3.4 Data analyses

The data were analysed separately for male and female bees using PASW Statistics 18 (i.e. SPSS 18.0) unless otherwise noted. Temperature effects on head capsule width, calyx branch area, MG density (lip and collar), and MG number (lip and collar) were investigated using ANOVAs with T1, T2-level (cool, medium, and warm), and T2-mode (constant vs. fluctuating) as fixed factors. The control group (Hut) was not included in the analyses but is presented in the figures as a reference for natural conditions.

In the cases of a non significant effect of T2-level, we conducted a power analysis (G-Power 3.1.2, freeware; Erdfelder et al. 1996) to estimate the type II error “ β ”. This analysis calculates the probability that we would have detected an effect of temperature (level) in *O. bicornis* if it was as large as in honeybees. The respective effect sizes for honeybees were determined based on the studies by Groh et al. (2004; derived from the values for the differences between 29 and 34.5°C in figure 2).

As in our previous study (Radmacher and Strohm 2011c), we wanted to compare the magnitude of temperature effects on the calyces in honeybees and *O. bicornis*. We determined the largest difference (in %) that was found between two (mean) values of calyx branch area, MG density, and MG number for a temperature difference of about 5°C. The larger mean value was defined as 100% and the difference (in %) to the lower (mean) value was calculated. This difference is further referred to as “relative temperature effect”. The data

for honeybees were derived from figure 2 in Groh et al. (2004). In *O. bicornis*, the data were analysed separately for both sexes as well as for constant and fluctuating temperature conditions. To test whether the effects on branch areas, MG densities and MG numbers for lip and collar that we found in *O. bicornis* are significantly smaller than those found in honeybees (Groh et al. 2004), we conducted one-sample T-tests. We used the values for the relative temperature effects in these variables in honeybees (derived from Groh et al. (2004) as described above) as the reference value. To obtain a sample of relative temperature effects for the respective variables (branch areas, MG densities and MG numbers for lip and collar) for *O. bicornis* (grouped with regard to sex and temperature mode (constant or fluctuating)), we used the same relative temperature effects as described above. However, now we did not calculate the difference between the larger and the lower mean value, but we calculated the relative differences between the larger mean value (that was defined as 100 % again) and the value for each individual that belongs to the group with the lower mean value. This enabled us to statistically compare the effects for honeybees and treatment groups of *O. bicornis* that were exposed to different temperatures for their whole developmental period (data from Radmacher and Strohm 2011c) and for post-larval development only (this study).

5.4 RESULTS

5.4.1 Immunofluorescence labelling of neuropils

Due to its affinity to dendritic F-actin, the fluorophore-conjugated phalloidin labelled all synaptic neuropils in the bee brain (Wieland 1987; Rössler et al. 2002; Frambach et al. 2004; Fig. 5.1A). Small circular structures (spherical, ellipsoidal or irregular in shape) were clearly visible in the intensely labelled calyces of the MB (Fig. 5.1B, C). These circular structures represent the MG since they are the labelled dendritic spines mostly from Kenyon cells (postsynaptic part) that surround a central, non-labelled bouton, formed by axons from projection neurons (presynaptic part). No conspicuous differences in gross brain morphology were noticed between temperature treatments or the sexes, except for the general size difference between males and females.

5.4.2 Effects of T1

In both sexes, T1 significantly affected the head capsule width (Males/Females: $F=51.79/23.16$, $df=1/1$, $p<0.001/<0.001$) and the calyx branch area ($F=12.46/11.49$, $df=1/1$, $p=0.001/0.002$).

Bees that were exposed to fluctuating T1 (10-25°C) until cocoon completion were larger and had larger calyces than bees that experienced constant T1 (17.5°C) during that phase (Fig. 5.2A, B). The MG density in the lip was not affected by T1 in both sexes (Males/Females: $F=1.08/1.67$, $df=1/1$, $p=0.306/0.205$; Fig. 5.2C). In the collar, the MG density tended to be slightly higher in 17.5°C than in 10-25°C, but this effect was significant only for females (Males/Females: $F=3.49/6.56$, $df=1/1$, $p=0.070/0.015$; Fig 5.2D). In males, the MG numbers in the lip and in the collar were slightly, but significantly higher in 10-25°C than in 17.5°C (lip/collar: $F=7.48/7.77$, $df=1/1$, $p=0.010/0.008$; Fig. 5.2E, F). In females, the MG numbers in both calyx subdivisions were not significantly affected by T1 (lip/collar: $F=2.18/3.199$, $df=1/1$, $p=0.149/0.082$; Fig. 5.2E, F).

5.4.3 Effects of T2

We found no significant effect of T2-mode (i.e. constant vs. fluctuating T2-temperatures) on any variable (i.e. calyx branch area, MG density (lip/collar), and MG number (lip/collar)) in both sexes (Males/Females: head capsule width: $F=0.015/0.118$, $df=1/1$, $p=0.903/0.733$; calyx branch area: $F=0.089/0.947$, $df=1/1$, $p=0.767/0.337$; lip MG density: $F=0.913/0.001$, $df=1/1$, $p=0.346/0.981$; collar MG density: $F=0.149/$, $df=1/1$, $p=0.702/$; lip MG number: $F=0.390/0.596$, $df=1/1$, $p=0.536/0.445$; collar MG number: $F=0.042/<0.001$, $df=1/1$, $p=0.838/0.994$).

T2-level (i.e. cool, medium or warm T2-temperatures) did not influence the head capsule width (Males/Females: $F=0.186/2.216$, $df=2/2$, $p=0.831/0.124$), but the calyx branch area ($F=3.321/3.795$, $df=2/2$, $p=0.048/0.032$) in both sexes, with a maximum area at medium temperatures (Fig. 5.3A, B). The MG densities in the lip (Males/Females: $F=1.654/1.18$, $df=2/2$, $p=0.206/0.319$) and in the collar ($F=0.225/1.152$, $df=2/2$, $p=0.800/0.328$) were not significantly affected by T2-level in both sexes (Fig. 5.3C, D). In males, T2-level significantly influenced the MG numbers in the lip ($F=4.557$, $df=2$, $p=0.017$) and the collar ($F=5.945$, $df=2$, $p=0.006$), tending to a maximum at medium temperatures at least under fluctuating conditions (Fig. 5.3E, F). In females, the MG numbers in both calyx subdivisions were not significantly affected by T2-level (lip/collar: $F=2.982/1.192$, $df=2/2$, $p=0.064/0.316$; Fig. 5.3E, F).

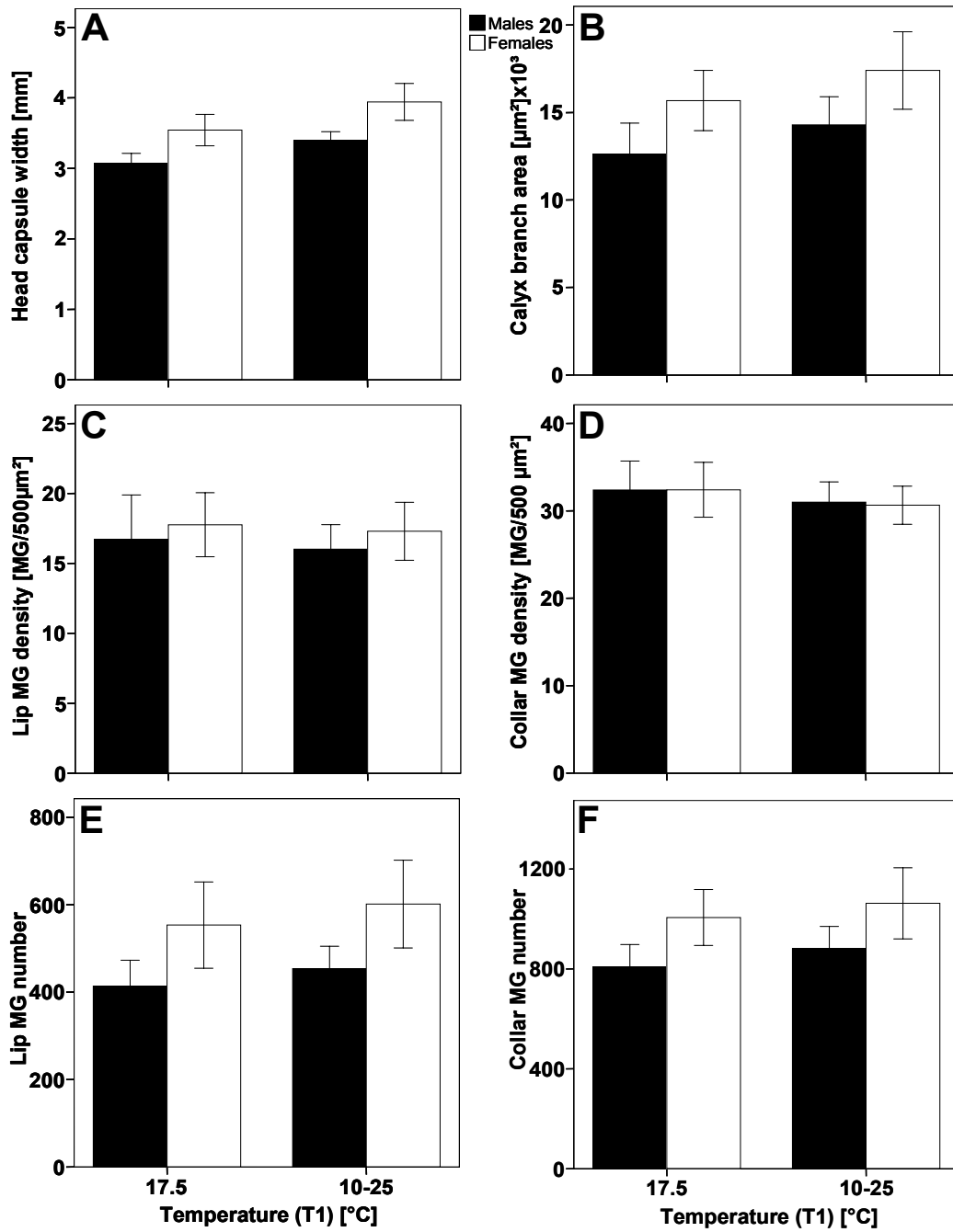


Figure 5.2: Effects of T1 (i.e. the temperature experienced during larval development) on head capsule width (A), calyx branch area (B), MG density in the lip (C) and in the collar (D), and MG number (i.e. extrapolated density to calyx branch area) in the lip (E) and the collar (F) in *O. bicornis*. The bars (black for males, white for females) represent the mean \pm SD. One fluctuating temperature treatment (10-25°C; $N_{\text{Males}}=29$, $N_{\text{Females}}=28$) and its constant mean temperature (17.5°C; $N_{\text{Males}}=27$, $N_{\text{Females}}=27$) were investigated.

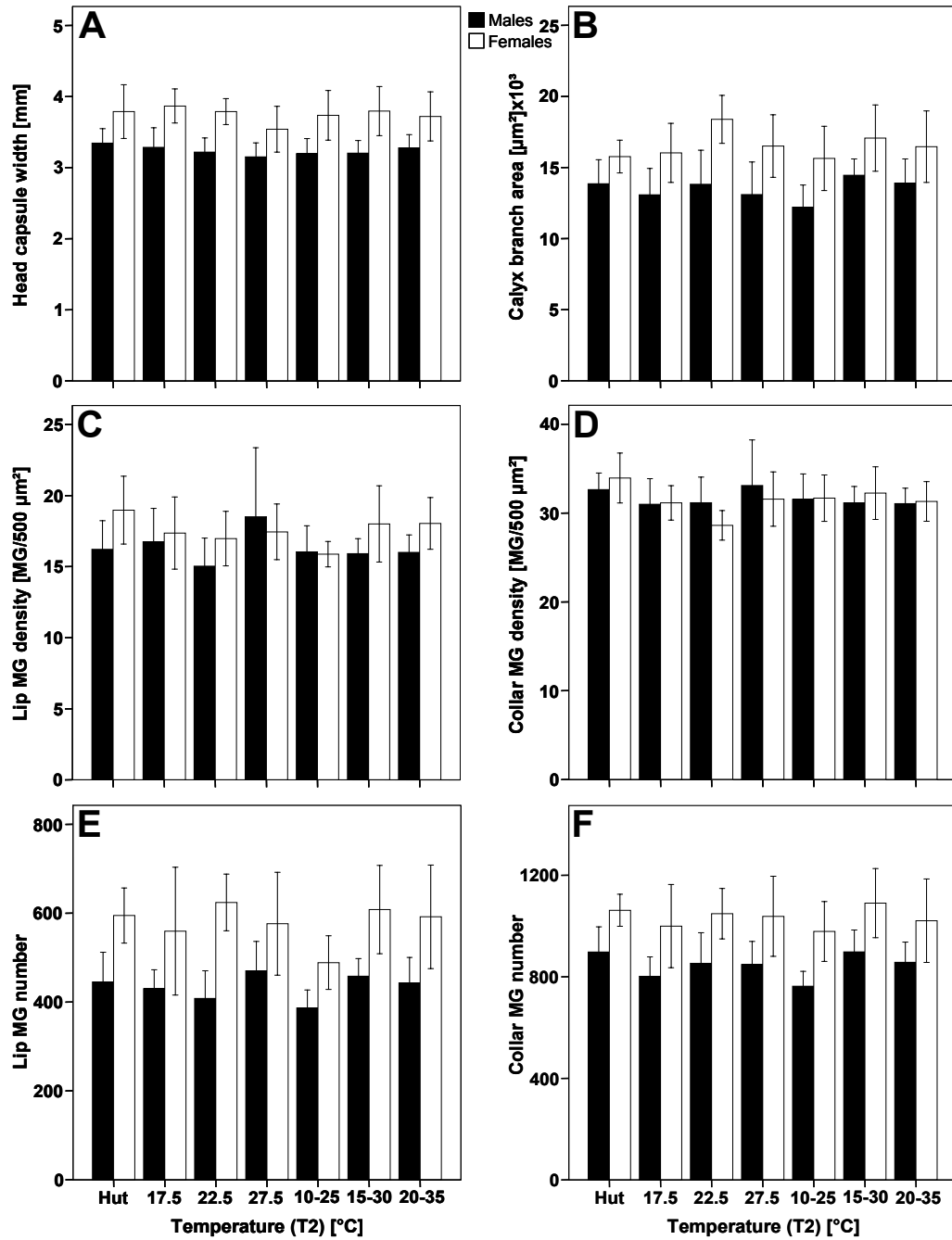


Figure 5.3: Effects of T2 (i.e. the temperature experienced during the prepupal and the pupal stage) on head capsule width (A), calyx branch area (B), MG density in the lip (C) and in the collar (D), and MG number (i.e. extrapolated density to calyx branch area) in the lip (E) and the collar (F) in *O. bicornis*. The bars (black for males, white for females) represent the mean \pm SD. Three fluctuating temperature regimes (10-25, 15-30, and 20-35°C), their constant mean temperatures (17.5, 22.5, and 27.5°C), and one control with natural temperature conditions (Hut) were investigated. Sample sizes (Males/Females) were: $N_{\text{Hut}}=8/8$, $N_{17.5^\circ\text{C}}=8/7$, $N_{22.5^\circ\text{C}}=8/8$, $N_{27.5^\circ\text{C}}=8/8$, $N_{10-25^\circ\text{C}}=8/8$, $N_{15-30^\circ\text{C}}=8/8$, $N_{20-35^\circ\text{C}}=8/8$.

Since we found no significant effect of T2-level on the MG density (in lip and collar) in both sexes and on the MG number (in lip and collar) in females, we conducted power analyses to assess the probability of false negative results. The estimated probability ($1-\beta$) of detecting temperature effects of a similar size as in honeybees (Groh et al. 2004) with our data would have been >0.999 in all tested cases.

5.4.4 Comparison of relative temperature effects

The relative temperature (i.e. T2-level) effects on calyx branch area, MG density (lip and collar), and MG number (lip and collar) in honeybees (data derived from Groh et al. 2004) and *O. bicornis* are listed in Table 5.1. For completeness, we included, besides the results from the present study in which the *O. bicornis* offspring were exposed to the respective temperature treatments only for post-larval development (called “PLD-*Osmia*” in the following), the data from our previous study (Radmacher and Strohm 2011c) in which *O. bicornis* experienced the experimental temperatures during their entire development (further referred to as “ED-*Osmia*”). The effects of constant temperatures on calyx branch area were smaller in PLD-*Osmia* than in ED-*Osmia*. In fluctuating temperatures, the relative temperature effects on calyx branch area were similar for the two *Osmia* groups. The relative temperature effects on the MG density in the lip and the collar were smaller in PLD-*Osmia* than in ED-*Osmia*, except for the lip MG density in males in constant temperatures whose relative temperature effects were similar in both groups. In contrast, the effects on MG number in the lip and the collar were generally larger in PLD-*Osmia* than in ED-*Osmia*, except for the collar MG number in both sexes in constant temperatures whose relative temperature effects were smaller in PLD-*Osmia*. However, the relative temperature effects on all variables were smaller in PLD-*Osmia* than in honey bees, particularly the effects on calyx branch area and overall MG numbers. Thus, the effects on total MG numbers were considerably and significantly larger in honeybees than in all experimental groups of *O. bicornis*.

Table 5.1: Comparison of relative temperature effects on honey bee workers (*Apis*, data derived from Fig. 2 in Groh et al. 2004) and *O. bicornis* (*Osmia*). For *O. bicornis*, we included the data resulting from this study on bees that experienced the experimental temperatures only during post-larval development (PLD-*Osmia*) as well as the data from our previous study (Radmacher and Strohm 2011c) in which bees were exposed to the experimental temperatures during their entire development (ED-*Osmia*). Listed are the largest differences (in %, see below) that we found between two mean values of each variable (i.e. calyx branch area, MG density in lip and collar, and MG number in lip and collar) for a temperature difference of about 5°C (4.5-5.5°C for honeybees). The higher value was defined as 100% and the difference to the lower value was calculated as the difference in % and defined as “relative temperature effect”. The data for *O. bicornis* were analysed separately for both sexes and for constant (const.) and fluctuating (fluct.) T2-temperatures (T). Significant differences (one-sample-t-tests; $p < 0.05$) between the relative temperature effects found in honeybees and in *O. bicornis* are indicated with an asterisk (*) next to the respective relative temperature effect in *O. bicornis*.

Variable	<i>Apis</i>	<u>PLD-<i>Osmia</i></u>				<u>ED-<i>Osmia</i></u>			
		♂ const. T	♀ const. T	♂ fluct. T	♀ fluct. T	♂ const. T	♀ const. T	♂ fluct. T	♀ fluct. T
Calyx branch area	43	5 *	13 *	16 *	8 *	24	36	13 *	13 *
Lip MG density	24	19	2 *	1 *	12 *	17	40 *	20	20
Collar MG density	24	6 *	9 *	1 *	3 *	24	21	12 *	7 *
Lip MG number	47	13 *	10 *	16 *	20 *	1 *	3 *	5 *	7 *
Collar MG number	59	6 *	5 *	15 *	10 *	11 *	17 *	5 *	8 *

5.5 DISCUSSION

5.5.1 Body size and neuropil size

The body size of bees (measured as adult head capsule width) was influenced by the temperature regime that they experienced during the larval phase (T1), resulting in larger bees in fluctuating temperatures (Fig. 5.2A). This result is consistent with our previous studies on *O. bicornis* in which the bees attained higher body weights in fluctuating (vs. constant) and cool (vs. medium and warm) temperatures that were applied during entire development (Radmacher and Strohm 2010, 2011a). However, in the present study the head capsule width was neither affected by T2-level nor by T2-mode, i.e. by the temperatures

experienced during the prepupal and the pupal stage (Fig. 5.3A). This suggests that the determination of adult body size in *Osmia* bees mainly takes place during the larval phase. It largely depends on the amount of consumed provision (Bosch and Vicens 2002; Radmacher and Strohm 2010) and is affected by the temperature conditions experienced during the larval phase (Radmacher and Strohm 2011a).

Neuropil size (i.e. calyx branch area) was affected by T1 and by T2-level. The effect of T1 might be interpreted as a simple function of the temperature effect on body size since larger bees usually have larger brains (Mares et al. 2005; Withers et al. 2008). However, with regard to T2-level the calyx branch area was maximal at medium temperatures though the head capsule width did not differ between the T2-treatments. This indicates that the neuropil size might be directly affected by temperature during the prepupal and the pupal stage (Fig. 5.3A, B). We found the same pattern (maximal neuropil size in medium temperatures) in ED-*Osmia* (Radmacher and Strohm 2011c), but the effect was stronger in ED-*Osmia* than in PLD-*Osmia* particularly in constant temperatures (Tab. 5.1). This was probably due to a combined effect of larval temperature on body size and of post-larval temperature on neuropil size in ED-*Osmia* that resulted in remarkably small calyces in the 27.5°C treatment (Radmacher and Strohm 2011c). By decoupling the temperature influences on larval and post-larval development in the present study, we obviously also decoupled (at least partly) the temperature influences on body size and neuropil size and, thus, reduced differences in body size. For honeybees, Groh et al. (2004) also reported maximal neuropil sizes in the medium (and optimal) rearing temperature of 34.5°C, particularly for the lip. Unfortunately, no data about the body size of the investigated honeybees were given, but since honeybees (at a certain locality) are generally much less variable in body size than *Osmia* bees (Ruttner 1992; e.g. Bosch and Vicens 2002), it might be assumed that the investigated honeybees did not considerably differ in body size. Taking these possible size effects into account, we enhanced the comparability of the data for neuropil sizes and total MG numbers in *O. bicornis* and honeybees by applying the respective temperature treatments only during post-larval development.

5.5.2 MG density and MG number

In ED-*Osmia*, we found reciprocal and, thus, compensatory temperature effects on neuropil size and MG density resulting in a more or less constant MG number (Radmacher and Strohm 2011c). However, in PLD-*Osmia* (this study), we found no significant temperature

(T2) effects on MG density though neuropil size was affected (see above). Moreover, the relative temperature effects on MG density were mostly smaller in PLD- than in ED-*Osmia* (Tab. 5.1). Thus, the compensation of temperature influences on neuropil size by reciprocal effects on MG density that we found in ED-*Osmia* seems not to work as properly when the bees were exposed to these temperatures only during post-larval development. As a consequence, the differences in neuropil size were more or less reflected in the total MG numbers in both sexes in PLD-*Osmia* and resulted in generally higher relative temperature effects than in ED-*Osmia* (Tab. 5.1) and in significant temperature effects on the total MG number in the lip and the collar in males (Fig. 5.2E, F; Fig. 5.3E, F). By contrast, there were no significant temperature (T1 and T2) effects on MG numbers in females, indicating that males, possibly due to their haploidy, might have a lower capability to compensate for environmental fluctuations than females as it has been reported for honeybees (Lerner 1970; Clarke et al. 1992). However, we found no indication for such a disadvantage for males in ED-*Osmia* (Radmacher and Strohm 2011c).

Our results suggest that the observed compensation of temperature effects on neuropil size by reciprocal effects on MG density in *O. bicornis* (Radmacher and Strohm 2011c) is mediated by the temperature experienced during larval development. ED-*Osmia* might have somehow acclimatized to the experimental temperatures during larval development, resulting in an appropriate compensation of temperature effects (probably during metamorphosis) on neuropil size. In general, temperature influences during early development on the thermal responses of subsequent developmental stages (others than the adult stage) seem to be hardly investigated. Steigenga and Fischer (2009) explored whether the larval rearing temperature influenced the survival of prepupae and pupae at different temperatures in the butterfly *Bicyclis anynana* and found no indication for acclimatization. However, in previous studies on *O. bicornis* we found that the temperature experienced during larval development influenced the thermal response in the duration of the prepupal stage, resulting in an appropriate adjustment of adult eclosion to the onset of wintering temperatures (Radmacher and Strohm 2011b). Thus, *O. bicornis* might use the temperature during larval development as a cue for the temperature conditions in their environment that allows the adjustment of several physiological processes in subsequent developmental stages to the prevailing conditions.

5.5.3 Comparison of temperature effects on *O. bicornis* and honeybees

Since there is evidence that the *O. bicornis* that were exposed to different temperatures

during their entire development (Radmacher and Strohm 2011c) had somehow acclimatized to the experimental temperatures during larval development, the data of the present study are probably more appropriate for a comparison of temperature effects between *O. bicornis* and honeybees (Groh et al. 2004). However, since we were not able to apply the experimental temperatures only during the pupal stage as it was the case in honeybees, we cannot completely exclude the possibility for some degree of acclimatization to the prevailing temperature during the prepupal stage in *O. bicornis*. Nevertheless, in this study we at least strongly reduced the possibility for any acclimatization.

The power analyses revealed that the non significant temperature (T2-level) effects on MG density (lip and collar) in both sexes and on MG number (lip and collar) in females were most likely not due to inadequate sample sizes or high variance since effects similar to those found in honeybees would have been detected with a very high probability. In addition, though the relative temperature effects on overall MG number in the lip and the collar were somewhat larger in PLD-*Osmia* than in ED-*Osmia*, they were still much smaller in *O. bicornis* than in honeybees. These findings support our hypothesis that solitary bees are adapted to fluctuating and changing temperature conditions and, thus, are generally less susceptible to variations in temperature during development than honeybees.

5.6 ACKNOWLEDGEMENTS

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CHAPTER 6

DOES DEVELOPMENTAL TEMPERATURE INFLUENCE THE LEARNING ABILITIES IN THE SOLITARY BEE *OSMIA BICORNIS* (HYMENOPTERA, MEGACHILIDAE)?

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6.1 SUMMARY

Learning and memory are essential for foraging performance in bees. In social honeybees, deviations from the optimal (nearly constant) pupal rearing temperature were shown to affect brain structure, behaviour, and learning and memory. In this study, we developed a visual learning paradigm for the solitary bee *Osmia bicornis* to test whether three fluctuating and three constant temperature regimes during pupal development might influence the learning abilities in this bee species. We found evidence for an innate preference for blue in *O. bicornis*, but the bees were generally able to overcome this innate preference by learning to associate a reward with yellow, also in a fast reversal learning task. We did not detect an effect of temperature during pupal development on the abilities for visual learning and fast reversal learning in *O. bicornis*. This is consistent with previous studies that revealed that, in *O. bicornis*, the brain structure is much less affected by developmental temperature than in honeybees. Our results support the hypothesis that, in contrast to honeybees, solitary bees are adapted and, thus, less susceptible to fluctuating temperatures during development.

6.2 INTRODUCTION

Learning and memory are of crucial importance for foraging performance, particularly in animals like bees that frequently travel between diverse flower patches and their nest (“central place foragers”) to provide food for them and their brood (Pyke et al. 1977; Hughes et al. 1992; Menzel and Müller 1996; Menzel 2001; Giurfa 2007; Dukas 2008; Raine and Chittka 2008). During the last decades, the cognitive abilities of the social honeybee have been intensely studied, revealing impressive learning and memory capacities some of which were previously only suspected in vertebrates (von Frisch 1914, 1927; Menzel et al. 1974; Gould 1984; Hammer and Menzel 1995; Menzel 2001; Zhang et al. 2005; Giurfa 2007; Avarguès-Weber et al. 2011). Though most studies concerning cognitive capabilities of insects were conducted with social insects, mostly honeybees, Menzel (2001) stated that “there is no reason to believe that the honeybee is in any way special in its cognitive capacities” among hymenopteran pollinators since the main requirements, namely finding food in an unpredictable environment and the navigation between nest site and feeding places, must be met by both social and solitary species (Menzel 2001; Amaya-Márquez and Wells 2008). However, compared to social Hymenoptera, learning and memory not to mention potential (environmental) influences on the cognitive capabilities of solitary bees have received little attention.

In this study, we wanted to investigate possible effects of temperature during pupal development on the cognitive abilities of the solitary bee *Osmia bicornis* (Linnaeus 1758, Hymenoptera, Megachilidae; formerly called *O. rufa*). The temperature experienced during development affects important life history traits, e.g. development time (Ratte 1984), body size (Atkinson 1994), and fecundity (e.g. Stillwell and Fox 2005), probably due to a direct influence on the biochemical processes during development (Johnston and Wilson 2006). In holometabolous insects, all tissues, including the central nervous system, are completely remodelled during metamorphosis that takes place in the pupal stage (Bauer 1904; Levine 1995; Gullan and Cranston 2010). Thus, the prevailing temperature during this phase might affect the physiological processes that underlie the formation of the adult brain and, thus, the behaviour and learning performance of bees. In honeybees, small deviations from the optimal rearing temperature during the pupal stage affected a) dancing behaviour and other behavioural traits (Tautz et al. 2003; Becher et al. 2009), b) learning and memory (Tautz et al. 2003; Jones et al. 2005), and c) the synaptic organization in the mushroom bodies (Groh et

al. 2004, 2006) that represent multisensory integration centres in the insect brain and play an important role for learning and memory (Menzel et al. 1994; Heisenberg 1998; Strausfeld et al. 1998, 2009). Though in our studies on the influence of developmental temperature on the synaptic organization in the brain of adult *O. bicornis* we detected no or only small effects (Radmacher and Strohm 2011c, d), there might be inconspicuous influences of temperature on the brain that do not manifest as observable structural changes but that might nevertheless influence the cognitive abilities and, thus, foraging performance of this important European fruit-tree pollinator.

For the investigation of learning and memory in *O. bicornis*, a suitable learning paradigm and test set-up had to be developed. A frequently employed tool for such purposes in social bees is classical conditioning to odours using the proboscis extension reflex (PER, a response to antennal contact with sucrose solution; Takeda 1961; Bitterman et al. 1983; Laloi et al 1999; Carew 2000; McCabe et al. 2007). However, despite extensive efforts we were not able to elicit the PER in *O. bicornis*. Thus, the first objective of this study was the development of a learning paradigm with a training and test protocol that allowed the investigation of learning abilities in this solitary bee. We chose a visual learning task in which the free-flying bees should learn to associate a reward (i.e. sucrose solution) with one of two colours (yellow or blue) through differential conditioning. Since preliminary tests revealed that *O. bicornis* might have an innate preference for blue compared with yellow, we investigated this possibility more in detail and accounted for it in the further development of the training and test protocol. The developed protocol for the test of a) learning success following a longer training period on one colour and of b) the ability for quick reversal learning in a subsequent short training period on the other colour was used for the second (and main) objective of this study: The investigation of temperature influences during pupal development (i.e. metamorphosis) on the cognitive abilities of a solitary bee. Since, in contrast to honeybees, solitary bees should be adapted to fluctuating and changing temperatures during development and since we did not find evidence for a strong effect of temperature on brain organization in the study species (see Radmacher and Strohm 2011a, b, c, d), we expected no remarkable influence of temperature treatment during pupal development on the learning performance in *O. bicornis*.

6.3 MATERIALS AND METHODS

6.3.1 Bees and temperature treatments

O. bicornis is an abundant univoltine and polylectic solitary bee in Central Europe that readily accepts various kinds of trap nests (Westrich 1989; Krunić and Stanisavljević 2006a). During the flight period that ranges from early April to mid-June, females build their linear nests in pre-existing cavities, provision the brood cells with pollen and some nectar and lay one egg per brood cell. The larvae hatch, consume their provisions, spin a cocoon in which they pass through a prepupal and a pupal stage and finally eclose as adults in autumn. The adult bees remain inside their cocoons, overwinter there and emerge the next spring when temperatures rise.

Bees were obtained from a free-ranging *O. bicornis* population nesting in trap nests made of dense styrofoam with transparent polycarbonate lids in an observation hut near the botanical garden of the University of Regensburg (for more details about the trap nests see Strohm et al. 2002; Radmacher and Strohm 2011a). During the peak nesting season in 2009 (14 to 26 April), brood cells with one to three day old eggs were separated from their nests, resealed with paper and transparent foil, and randomly allocated to one of two temperature treatments (constant 17.5°C and fluctuating 10-25°C) for the development until cocoon completion. On the day of cocoon completion, the individual cocoons were removed from their brood cells and randomly allocated to one of seven temperature treatments for the development inside the cocoon, i.e. the prepupal and the pupal stage that includes metamorphosis. The seven temperature treatments were: Three fluctuating temperature regimes (10-25, 15-30, and 20-35°C), three constant temperatures that represented the mean temperatures of the fluctuating regimes (17.5, 22.5, and 27.5°C), and one control group (“Treatment control”) that was exposed to the natural temperature conditions in the observation hut. After a pre-wintering period that corresponded to about one third of the development time from egg to adult, the cocoons of the different temperature groups were transferred to constant 17.5°C for one week and then to constant 4°C for wintering. For more details about the temperature treatments and pre-wintering see Radmacher and Strohm (2011a, b, d).

For the development of the training and test methods and the investigation of potential innate colour preferences, we also used bees that experienced the natural temperature conditions inside their nests in the observation hut during their entire development, the

pre-wintering phase, and at least the largest part of overwintering (further referred to as “Natural control” group). Some bees that naturally emerged in April 2010 in the observation hut were captured directly after emergence from the cocoon and used for the preliminary trials. For the investigation of innate colour preferences, we needed such bees also after the natural emergence period. Therefore, we transferred 12 entire nests from the hut to a climate chamber with constant 4°C on 18 February 2010 to delay emergence. Cocoons from these nests were incubated to initiate emergence as needed (see below).

6.3.2 Incubation and colour training

Between the beginning of April and the beginning of June 2010, bees were incubated, trained and tested in a laboratory illuminated by 48 fluorescent tubes (HI LITE, F3678 Cool White) from 6:00 am to 10:00 pm. Room temperature usually ranged from 23-27°C, never falling below 20°C or exceeding 29°C. To induce emergence, the cocoons were individually transferred from the overwintering conditions (constant 4°C in a dark climate chamber) into small polystyrol vials (height: 80 mm, diameter: 35 mm; with a foam plug) that were stored in the laboratory. Several times a day, the vials were checked for emerged bees that were individually transferred into small flight cages (one bee per cage) made of a wooden frame covered with gauze, except for the open bottom (length x width x height: 40.5 x 23.5 x 23.5 cm). The cages were put with the bottom on tables that were covered with blank white paper. In order to eliminate possible chemical markings, the paper was exchanged after a training- and test-phase before the next bee was transferred to a cage. Since for the final tests we used only females, all males were released after the preliminary tests had been finished.

The training phase started with the transfer of a recently (up to ½ day) emerged bee to a cage where two artificial flowers were offered: One blue and one yellow flower that were placed about 3-4 cm apart from each other. An artificial training flower consisted of a small bloc of dense styrofoam (about 2.5 x 2.5 x 2.0 cm, light green) with a PCR-tube without cap in the centre of the upper side (volume: 0.2 ml; diameter: about 6 mm) as reservoir for the rewards. The bloc was covered with a blue or yellow cardboard disc (diameter: 3 cm) with a central hole for the reward reservoir (Fig. 6.1). One colour was rewarding, the other was not. During the first 2-3 days of the training phase in the cage, the rewarding flower was filled with diluted honey (about 20 % honey in distilled water), providing an olfactory stimulus for the bees that should facilitate finding the food. For the remaining 4-5 days of the training phase, the reward reservoir and the cardboard disk were renewed and filled with an odourless

sugar solution (about 20 % sucrose in distilled water) to provide only the colour stimulus. While the rewarding flower was refilled at least once a day so that it never ran dry, the non-rewarding flower was always empty. The positions of the rewarding and the non-rewarding flower were exchanged daily to assure that the bees were learning the rewarding colour rather than a rewarding location in the cage.

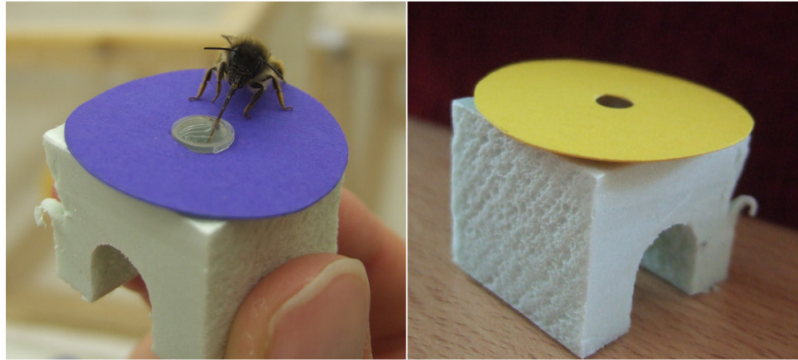


Figure 6.1: Artificial flowers used for associative colour training and tests. On the left: An *O. bicornis* female on a blue flower filled with sucrose solution. On the right: A yellow flower. (photos: A. Miglo)

After seven days of training on a rewarding colour (further referred to as “TC1” for training colour 1), test trial 1 was conducted in the morning of the 8th day. Since the bee should be motivated to search for food when the test was conducted, both artificial flowers were removed from 5:30-6:30 pm on the 7th day to starve the bee overnight. Immediately after test trial 1 (for a detailed test trial description see below), two new artificial training flowers were offered to the bee. However, the colours of the rewarding (i.e. filled with sugar solution) and the non-rewarding flower were reversed with regard to TC1. The rewarding colour in this second short training phase is further referred to as “TC2”. To prevent a too long starvation phase, the bee was observed and, if necessary, animated to crawl on the rewarding flower so that it could ingest some food. From 5:30-6:30 pm of the 8th day, the training flowers were removed again to starve the bee for test trial 2 that was conducted in the morning of the 9th day. After test trial 2, the bee was released.

Since the motivation for food search in the test trials was usually very high (see 6.4.1), longer starvation phases of about one day as were used by Vorel (2010) for *O. lignaria* and *Megachile rotundata* were not necessary and, moreover, seemed not to be recommendable for *O. bicornis* kept under the given conditions: Preliminary tests revealed elevated mortality when the training flowers were removed about noon on the day before a test trial.

6.3.3 Test trials

All test trials were conducted between 8:00 and 11:00 am. When a bee that should be tested started activity and showed signs of food searching behaviour (i.e. crawling or flying around in the cage), two artificial test flowers were put in the middle of the cage (again about 3 to 4 cm apart from each other). Both test flowers were empty, thus, no reward was provided in the test trials. The coloured cardboard disks of the test flowers were replaced by new ones for each test trial to prevent potential influences of scent marks of previous flower visitors (see e.g. Free and Williams 1983; Goulson et al. 1998; Gilbert et al. 2001; Yokoi et al. 2007; Yokoi and Fujisaki 2009) on the choice of the tested bee. The colour of the test flower on which the bee landed (or crawled) first and showed food searching behaviour (i.e. intensive inspection of the cardboard disc with the antennae, mostly extending the proboscis) was recorded as the “chosen colour”. Hovering around an artificial flower or crawling only on the sides of the styrofoam bloc were not regarded as sufficient for a positive choice. After a recorded colour choice, the test trial was finished and the test flowers were removed.

6.3.4 Investigation of innate colour preferences

A test for an actual innate colour preference would have required testing freshly emerged bees that have never seen a flower before. However, since freshly emerged *O. bicornis* were not motivated to search for food for an unpredictable time span, we chose an alternative approach to assess an innate colour preference. We assumed that the bees would learn a preferred colour faster or better than non-preferred colours as it has been shown for honeybees and bumblebees (Heinrich et al. 1977; Giurfa et al. 1995). In order to test for a potential innate preference for blue in *O. bicornis*, we used 20 females from the “Natural control”-group and 22 females from the “Treatment control”-group. One half was trained on TC1= yellow, the other half on TC1= blue. After test trial 1, bees were trained on the other colour TC2 (i.e. if TC1=yellow, TC2= blue and vice versa) for one day, followed by test trial 2. For the “Treatment control”-group, one bee that was trained to TC2=yellow died on 8th day and, thus, could not complete test trial 2.

6.3.5 Effect of developmental temperature on learning performance

The main goal of this study was to investigate potential temperature influences during development, particularly during the pupal stage, on the learning abilities of *O. bicornis*. Since it was not possible to obtain sufficient sample sizes for bees from each temperature treatment trained on both TC1-colours and since the bees might have an innate preference

for blue, we decided to use only yellow as TC1 based on the following assumption: If there was an innate preference for blue in *O. bicornis*, learning yellow as rewarding colour and finally choosing the yellow colour in the test would reflect more clearly a real cognitive effort than following the innate preference for blue that probably would have been simply a bit strengthened by a training on blue. Thus, following the general training- and test-protocol described above, 10-11 bee females from every temperature treatment ($N_{17.5^{\circ}\text{C}}=11$, $N_{22.5^{\circ}\text{C}}=10$, $N_{27.5^{\circ}\text{C}}=11$, $N_{10-25^{\circ}\text{C}}=11$, $N_{15-30^{\circ}\text{C}}=11$, $N_{20-35^{\circ}\text{C}}=11$) were trained on TC1=yellow, subjected to test trial 1, then trained on TC2=blue and subjected to test trial 2.

6.3.6 Data analyses

A potential effect of temperature treatment on the timing of emergence after onset of incubation was investigated with a Kruskal-Wallis test using the software PAST (Hammer et al. 2001). All other statistical analyses (described below) were performed using the software BiAS 8.2. To test for an innate preference for blue, the proportions of bees that chose the “right” colour (that was rewarded during the training period; TC1 for test trial 1, TC2 for test trial 2) were compared between bees that were trained on yellow or blue using Fisher’s exact test (two-tailed). This test was separately conducted for the “Natural control”-group and the “Treatment control”-group and for test trial 1 and test trial 2. To investigate reversal learning ability with regard to both colours, the proportions of bees that chose blue and yellow were compared between test trial 1 and test trial 2 with Fisher’s exact test (two-tailed) that was separately conducted for both control groups and for the training colour groups (i.e. TC1=yellow/TC2=blue and TC1=blue/TC2=yellow).

Potential differences in learning ability of bees from the different temperature treatments were investigated using χ^2 -contingency tables in which the proportions of bees that chose yellow (“right” colour in test trial 1) or blue (“right” colour in test trial 2) were compared between temperature groups. The bees from the “Treatment control”-group and the “Natural control”-group that were trained on TC1=yellow in the course of the investigation of an innate colour preference (see 6.3.4) were included in the analyses and the figures. They serve as a reference for completely natural temperature conditions during entire development (“Natural control”) and as control group that experienced the same manipulations during development like the bees of the (other) temperature treatments (“Treatment control”). The contingency tables were conducted separately for test trial 1 and test trial 2.

6.4 RESULTS

6.4.1 Bee behaviour in the training and test set-up

The timing of emergence after onset of incubation did not significantly differ between the temperature treatments ($H=10.33$, $p=0.1115$). Bees usually emerged after 2-4 days (minimum: $\frac{1}{2}$ day, maximum: 11 days) of incubation at laboratory room temperature.

After a phase of up to one day in which the freshly emerged bees tried to escape from the cage by searching for holes and biting into the gauze, they obviously habituated to the situation in the cage, found the food in the rewarding flowers, and flew and crawled freely around in the cages during their activity phases. Bees were inactive at night and also showed activity pauses during the day, particularly in the afternoon. During inactivity, they sat more or less motionless in a corner of the cage or on/in the artificial flowers. Ten out of overall 117 bees that were introduced in the cages (i.e. 8.5 %) died during the training phase(s), but no one died during a starvation phase.

In the test trials, the motivation of the partly starved bees to search for food was usually high: Most bees landed or crawled on an artificial flower and showed searching behaviour within 5 minutes after the introduction of the test flowers into the cage. Only two bees (of overall 107 tested bees; 1.9 %) ignored the flowers during the first 20 minutes after the introduction of the test flowers for test trial 1 on the 8th day. In these two cases, the training flowers were introduced again and test trial 1 was repeated on the 9th day when the bees reacted promptly within 5 minutes. On the 10th day, these two bees completed test trial 2.

In the test trials, most bees flew/crawled specifically to a distinct flower and ignored the other test flower. Only a few bees hovered or crawled shortly around both flowers before they chose one for landing and inspection. Since bees could not find any food in the test flowers, they usually took off after a short inspection of the chosen test flower.

6.4.2 Innate preference for blue and reversal learning ability

In the “Natural control”-group, 90 % of the bees with TC1=blue, but only 50 % of the bees with TC1=yellow chose the right colour in test trial 1 (Fig. 6.2A). However, this trend was not significant (Fisher’s $p=0.141$). In the “Treatment-control”-group, all bees with TC1=blue, but less than half the bees with TC1=yellow chose the right colour in test trial 1 (Fig. 6.2B);

this difference was significant (Fisher's $p=0.012$). We found no significant differences in the proportions of bees that chose the right colour in test trial 2, neither in the “Natural control”-group (Fisher's $p=0.656$), nor in the “treatment control”-group (Fischer's $p=0.198$). In both groups, more bees chose the blue test flower in test trial 2, regardless of the training colour TC2 (Fig. 6.2C, D).

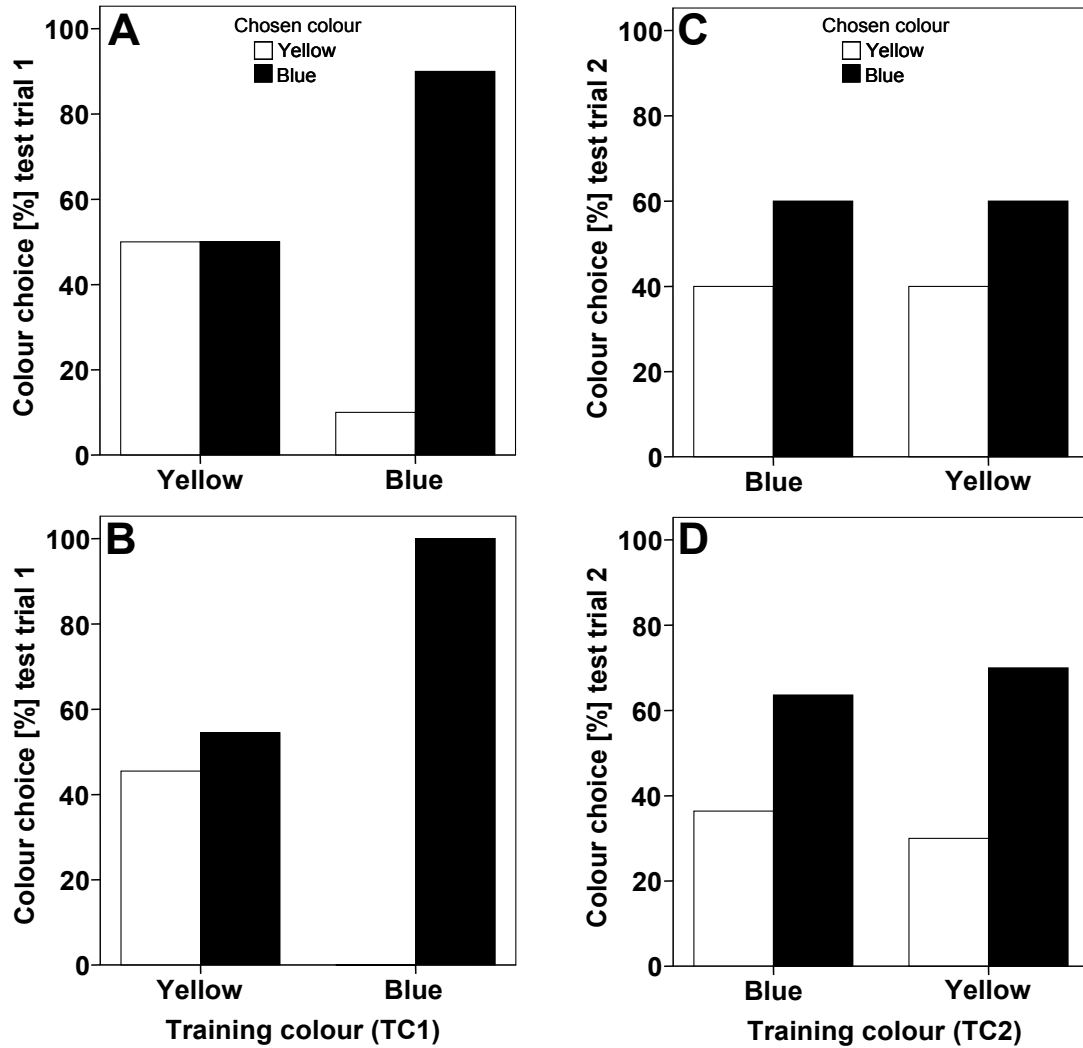


Figure 6.2: Investigation of a preference for blue in *O. bicornis* females. Bees that experienced natural temperature conditions during their entire lifetime until emergence (“Natural control”-group; A and C) and bees that were manipulated in the same way as the other temperature treatment groups but experienced natural temperature conditions during pupal development (“Treatment control”-group; B and D) were trained on yellow or blue (TC1) and tested for their first choice after one week (test trial 1, A and B; Sample sizes: “Natural control”-group (A) $N_{TC1=yellow}=10$, $N_{TC1=blue}=10$; “Treatment control”-group (B) $N_{TC1=yellow}=11$, $N_{TC1=blue}=11$). Then they were trained on the other colour (TC2) for one day and tested for their first colour choice again (test trial 2, C and D; Sample sizes: “Natural control”-group (C) $N_{TC2=blue}=10$, $N_{TC2=yellow}=10$; “Treatment control”-group (D) $N_{TC2=blue}=11$, $N_{TC2=yellow}=10$). Given are the proportions (in %) of bees of a TC-group that chose yellow (white bars) and blue (black bars) in the respective test.

We found some indications for reversal learning ability with regard to both colours in both control groups: In the training colour group TC1=yellow/TC2=blue, slightly more bees chose blue in test trial 2 than in test trial 1 and in the TC1=blue/TC2=yellow-group, more bees chose yellow in test trial 2 than in test trial 1 (Fig 6.2 A, C and B, D). However, these trends were not significant (Natural control: TC1=yellow/TC2=blue: Fisher's $p=1.0$, TC1=blue/TC2=yellow: Fisher's $p=0.303$; Treatment control: TC1=yellow/TC2=blue: Fisher's $p=1.0$, TC1=blue/TC2=yellow: Fisher's $p=0.214$).

6.4.3 Effect of developmental temperature on learning performance

In test trial 1, about half the bees (50-60%) in all temperature treatments chose yellow, i.e. the right colour for all bees in test trial 1 in this experiment (Fig. 6.3A). In test trial 2, more than half the bees (60-80 %) in all temperature treatments chose blue, i.e. the right colour in Test 2 (Fig. 6.3B). However, there were no significant differences between the temperature treatments in the proportion of bees that chose the right colour in test trial 1 ($\chi^2=0.9366$, $df=7$, $p=0.996$) and in test trial 2 ($\chi^2=3.4046$, $df=7$, $p=0.845$).

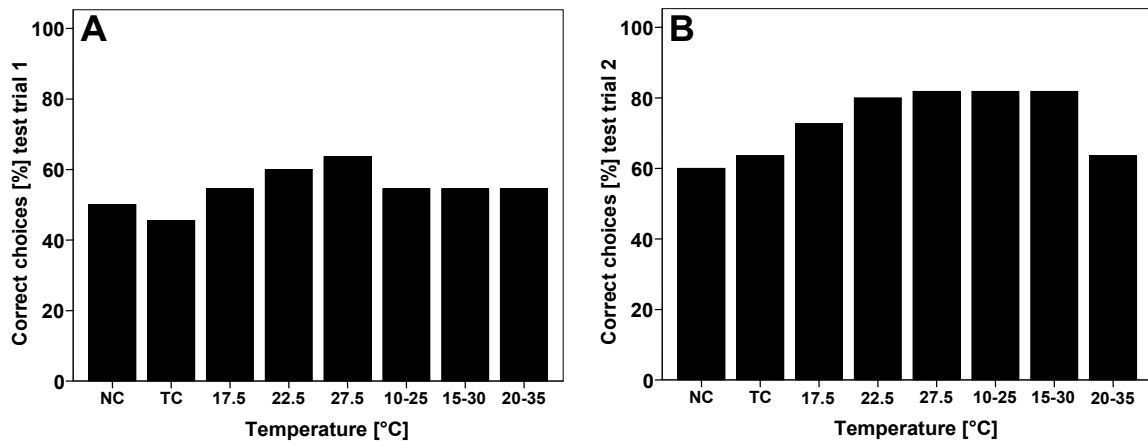


Figure 6.3: Influences of temperature during pupal development on the learning success of *O. bicornis* females. Bees that were exposed to different temperature treatments during development were trained on yellow for one week and then tested for their first colour choice (test trial 1, A). Then they were trained on blue for one day and tested for their first colour choice again (test trial 2, B). Given are the proportions of bees that chose the “right” colour in the respective test trial (i.e. yellow in test trial 1 (A) and blue in test trial 2 (B)). The “Natural control”-group (NC) had experienced natural temperature conditions during entire life. The “Treatment control”-group (TC) was manipulated in the same way as the other temperature treatment groups but experienced natural temperature conditions during pupal development. Sample sizes were: $N_{NC}=10$, $N_{TC}=11$, $N_{17.5^{\circ}C}=11$, $N_{22.5^{\circ}C}=10$, $N_{27.5^{\circ}C}=11$, $N_{10-25^{\circ}C}=11$, $N_{15-30^{\circ}C}=11$, $N_{20-35^{\circ}C}=11$.

6.5 DISCUSSION

6.5.1 Learning paradigm, training and test set-up

Preliminary tests revealed that the most prominent tool for the investigation of cognitive capabilities in Hymenoptera, i.e. conditioning of the PER, was not applicable in *O. bicornis* since these bees did not extend their proboscis in response to antennal stimulation with sucrose solution, in contrast to honeybees (e.g. Bitterman et al. 1983), bumblebees (e.g. Laloi et al. 1999), stingless bees (McCabe et al. 2007) and eusocial wasps (Vorel and Pitts-Singer 2010). This finding is consistent with the results of Vorel and Pitts-Singer (2010) who also failed to elicit the PER in other Megachilidae. This suggests that these solitary bees might generally not exhibit this reflex (but see Anfora et al. 2010). As a consequence, we had to develop a suitable learning paradigm for *O. bicornis*, though the comparability of the revealed learning abilities with other (bee) species would be reduced. However, since our main goal was the comparison of the learning performance between groups of the same species (*O. bicornis*) that differ in developmental temperature, this approach was sufficient for our purposes. In contrast to Vorel (2010) who developed an olfactory conditioning method for megachilid bees, we chose a visual learning task in a foraging context as it was done for other non-social insects like butterflies and flies (Fukushi 1989; Goulson and Cory 1993; Weiss 1995; Kelber 1996; Kandori et al. 2009) as well as solitary bees (Dukas and Real 1991; Perez and Waddington 1996; Amaya-Márquez et al. 2008). A visual learning task seemed to be advantageous since in an olfactory learning task we would have been hardly able to exclude potential conditioning influences of larval food on the choice of the adult bee (Dobson 1987; Blackiston et al. 2008; Vorel 2010) which are very unlikely for visual (i.e. colour) cues since the bees develop in the dark. Menzel and colleagues (Menzel et al. 1988; Steinmann and Menzel 1990) already investigated visual learning in *O. bicornis*. However, in these studies the bees learned colours in the context of nest-finding but not in a food-searching context – and visual learning may be context-dependent in bees (Menzel 1985; Avarguès-Weber et al. 2011). To our knowledge, this study was the first attempt to investigate visual learning in *O. bicornis* in a foraging context.

In general, the chosen conditions during the incubation and training phases and the duration of starvation phase seemed to be suitable for *O. bicornis* since the mortality of bees was low. Consistent with investigations of spectral sensitivity, colour vision, and colour learning in a nest-finding context in *O. bicornis* (Menzel et al. 1988; Steinmann and Menzel 1990), in our

experiments the bees seemed to be able to discriminate well between the yellow and blue colour under the light conditions in the arena. Though there was some evidence for an innate preference for blue (see below), preliminary tests indicated that this innate preference could be overcome by learning when the training phase in which the bees learned to associate the food reward with yellow was about one week. This finding accords to Menzel et al. (1988) who stated that “*Osmia* does not show a strong tendency to use particular colours which cannot be overcome by training”.

6.5.2 Innate preference for blue

Preliminary observations as well as other studies (Menzel et al. 1988; Amaya-Márquez et al. 2008) suggested that *Osmia* bees probably have an innate preference for blue – compared to yellow, green or white. Innate preferences for a certain colour or colour range are common among flower visitors: Honeybees, bumblebees and other Apidae often (but not always) preferred blue over yellow or other colours (Müller 1881; Giurfa et al. 1995; Lunau and Maier 1995; Lunau et al. 1996; Perez and Waddington 1996; Gumbert 2000; Simmonds and Plowright 2004; Stephen and Rao 2005; Amaya-Márquez et al. 2008), some flies showed a preference for yellow (Fukushi 1989; Lunau and Maier 1995), and the colour preference of butterflies varied among species (Lunau and Maier 1995; Kelber 1996; Weiss 1997; Kandori et al. 2009). It should be mentioned that a preference for blue (compared to yellow) might be a preference for the stronger contrast with the background rather than for the blue spectrum itself (e.g. Lunau et al. 1996). We cannot exclude this possibility in our investigations; however, it does not play a role for the interpretation of our results if *O. bicornis* preferred the blue colour itself or the stronger contrast to the background. For simplicity, we continue to use the terms “colour preference” or a “preference for blue”.

The high learning success of bees with TC1=blue that was demonstrated in test trial 1 (Fig. 6.2A, B) and the higher proportion of bees that chose blue in test trial 2 irrespective of the training colour (TC2) supports the assumption of an innate preference for blue in *O. bicornis*. However, since about half of the bees with TC1=yellow chose the right colour in test trial 1 and 30-40 % of the bees chose yellow in test trial 2 (irrespective of TC2; Fig. 6.2C, D), our results indicate that *O. bicornis* is able to overcome this innate preference by associative learning in a longer (i.e. one week) training phase and even in a short training phase for reversal learning. In general, fast reversal learning seems to be possible for both colours – the preferred blue and the non-preferred yellow – since more bees chose the respective

“right” colour (for test trial 2) in test trial 2 than in test trial 1, particularly in the TC1=blue/TC2=yellow-groups.

6.5.3 Temperature effects on learning abilities

In the main experiment, we wanted to compare a) the ability to overcome the innate preference for blue by learning to associate a food reward with yellow and b) the ability of fast reversal learning between groups of bees that were exposed to different temperatures during the prepupal and the pupal stage. The ratio of about 50 to 50 % for the choice of yellow and blue in test trial 1 in all temperature treatments (Fig. 6.3A) might suggest that the bees chose the colour rather randomly than based on learning in this test. This would mean that the developed training and test methods might have been inappropriate for our purposes. However, the behaviour of the bees during the training phases and tests suggested that the choice of an individual bee was not random, but that some bees (i.e. about the half) really learned to associate the yellow colour with a food reward and made their choice in test trial 1 based on learning while other bees were obviously not able to overcome their innate preference for blue under the given training conditions. Almost all the bees that chose blue in test trial 1 also chose blue in test trial 2 – in these cases, the choice of the “right” colour in test trial 2 might be attributed to the persistence of the innate preference for blue rather than to successful reversal learning. However, as indicated by the fact that the proportion of bees that chose blue in test trial 2 was larger than the proportion of bees that chose blue in test trial 1 for all groups (Fig. 6.3 A, B), some of the bees that had learned to associate yellow with the food reward in the first (long) training phase chose blue in test trial 2, probably due to fast reversal learning during the second (short) training phase. Thus, the results for the bees of the temperature treatments additionally support the conclusion (that were drawn from the investigation of both control groups, see above) that *O. bicornis* has generally shown the ability for visual learning and fast reversal learning in a foraging context. However, since we found no significant differences between the temperature treatments in the proportion of bees that chose the right colour in test trial 1 or in test trial 2, our results suggests that the temperature experienced during pupal development did not affect the visual learning abilities of *O. bicornis*.

Whereas visual learning in a foraging context in general has been investigated in detail in social bees and more recently also in some solitary bee species (Menzel 1967; Menzel et al. 1974; Heinrich et al. 1977; Dukas and Real 1991; Perez and Waddington 1996;

Amaya-Márquez et al. 2008; Avarguès-Weber et al. 2011), the ability for reversal learning in conditioned flower visitors received less attention though it might play an important role for all generalists that are confronted with a changing flower supply during their lifetime. Reversal learning abilities regarding the association of distinct colours with a food reward have been reported for the social honeybees and bumblebees (Menzel 1969; Heinrich et al. 1977; Meineke 1978) as well as for non-hymenopteran insects like butterflies (Goulson and Cory 1993; Kelber 1996), but they seem to be hardly investigated in solitary bees.

Though temperature experienced during development affects many fitness-relevant life history traits in insects in general (Ratte 1984; Atkinson 1994; Johnston and Wilson 2006), its influence on the learning abilities and, thus, foraging performance has only been investigated in the social honeybees so far. Honeybees actively control the temperature in their nest (Jones and Oldroyd 2006). Deviations from the optimal rearing temperature of about 34.5-35.5°C during pupal development were shown to affect the synaptic organization in the mushroom bodies (Groh et al. 2004, 2006) that play an important role for learning and memory in insects (Menzel et al. 1994; Heisenberg 1998; Strausfeld et al. 1998, 2009) as well as olfactory learning and memory abilities (Tautz et al. 2003; Jones et al. 2005) in adult honeybees. In contrast, the offspring of solitary bees like *O. bicornis* are more or less exposed to the fluctuating and changing ambient temperatures. Therefore, these bees should be adapted to changing temperatures and, thus, they should be less susceptible to temperature influences during development. According to this hypothesis, we found no or only weak effects of temperature during pupal development on the synaptic organization in the mushroom bodies (Radmacher and Strohm 2011d). The results of this study on visual learning and reversal learning abilities of adult *O. bicornis* females are consistent with this lack of effects of temperature on brain structure. However, since we detected small effects of temperature during pupal development on the overall number of distinct synaptic complexes in the mushroom bodies of *O. bicornis* males (Radmacher and Strohm 2011d), the investigation of learning and relearning abilities in male bees might provide further insights about potential temperature influences on the brain and cognitive capacities in insects.

6.6 ACKNOWLEDGEMENTS

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CHAPTER 7

GENERAL DISCUSSION

7.1 Temperature effects on body size and development time

Temperature influences during development resulted in significant differences in body size and development time and, thus, in developmental plasticity in these traits in *O. bicornis*. As mentioned in chapter 5, adult body size of *Osmia* bees seems to be mainly determined during larval development: It largely depends on the amount of consumed pollen provision (Bosch and Vicens 2002; Radmacher and Strohm 2010) and is remarkably affected by temperature during development, particularly under constant temperature conditions (Radmacher and Strohm 2010; chapter 2). The body size of *O. bicornis* decreased with increasing temperature, according to the so called temperature-size rule that has shown to be valid for most (but not all; see e.g. Schroeder and Lawson 1992; Mousseau 1997; Walters and Hassall 2006) ectotherms (Atkinson 1994; van der Have and de Jong 1996; Kingsolver and Huey 2008). In insects, body size is usually positively correlated with reproductive success (e.g. Thornhill and Alcock 1983; Kasule 1991; Honěk 1993; Strohm and Linsenmair 1997; Sokolovska et al. 2000). In bees (including *O. bicornis* and other megachilid species), advantages for larger individuals in mating success, fecundity and foraging efficiency have been frequently reported (Stephen and Osgood 1965; Alcock et al. 1977; Torchio and Tepedino 1980; Severinghaus et al. 1981; Sugiura and Maeta 1989; Johnson 1990; Stone 1994; Kim 1997; Smith et al. 2008; Seidelmann et al. 2010). Thus, an increase in developmental temperature might result in a decrease in body size and, thus, in a decrease in reproductive success in (solitary) bees. However, in several studies on solitary bees (including *O. bicornis*) no advantages for larger individuals with regard to reproductive success were found (Tepedino and Torchio 1982; Frohlich and Tepedino 1986; Johnson 1990; Sugiura 1994; Alcock et al. 2006; Bosch and Vicens 2006; Strohm and Liebig 2008). Moreover, under fluctuating temperature conditions (as are prevailing in nature) the prepupal weight in *O. bicornis* was only slightly reduced with increasing overall temperatures (chapter 2). Though one should take into account that considerable weight loss can occur during the pupal stage (Bosch and Vicens 2002; Sgolastra et al. 2010) and that we cannot exclude that this pupal weight loss

could be affected by (fluctuating) temperature, we tentatively conclude that, under fluctuating conditions, an overall warming of several degrees during development - as it presumably can be expected due to climate change – would probably have no severe negative effects on the final body size of *O. bicornis* at the time of adult eclosion.

However, the life cycle of *Osmia* bees is not yet completed with adult eclosion. The adult bees stay inside their cocoons and experience a phase of pre-wintering until temperature drops and bees enter the wintering phase. Larger individuals are more likely to survive the winter than smaller bees (Tepedino and Torchio 1982; Kim and Thorp 2001; Bosch and Kemp 2004), possibly due to a larger relative fat content with increasing body size (Strohm 2000; Hahn and Denlinger 2007). During pre-wintering and wintering, the bees completely rely on energy reserves in their body tissue (fat body). Moreover, the bees still need sufficient energy reserves for post-diapause activities like emergence from the cocoon and the nest. Thus, economising energy reserves seems to be crucial for overwintering survival and fitness. Therefore, the bees usually lower their metabolism (indicated by lowered respiration rates) shortly after adult eclosion (Bosch et al. 2010, Sgolastra et al. 2010). However, although lowered, metabolism remains responsive to temperature in (diapausing) insects (Hahn and Denlinger 2007) and despite a lowered respiration rate, rapid weight loss occurred in diapausing *O. lignaria* at elevated temperatures (Sgolastra et al. 2010). Thus, elevated (constant) temperatures during pre-wintering and wintering resulted in increased weight loss and mortality in *Osmia* bees, probably due to fat body depletion (Bosch and Kemp 2003, 2004; Sgolastra et al. 2010).

In *O. lignaria* and *O. cornuta*, particularly the duration and temperature conditions of the pre-wintering phase (defined as the time between adult eclosion and the onset of wintering temperatures) seem to play an important role for weight loss and survival (Bosch and Kemp 2004; Bosch et al. 2000, 2010). Our results (chapter 2 and 3) confirmed this crucial role of the pre-wintering phase for *O. bicornis*. Almost all developmental stages of *Osmia* bees obviously follow the often observed patterns that increasing and fluctuating (vs. constant) temperatures result in an acceleration of development (Bosch and Kemp 2000; Bosch et al. 2000; Kemp and Bosch 2005; chapter 2 and 3), probably due to the acceleration of biochemical processes at higher temperatures and the non-linear but (in part) exponential relationship between temperature and development rate (Kaufmann 1932; Beck 1983; Ratte 1984; Cossins and Bowler 1987; Johnston and Wilson 2006). In 2008, when we exposed *O. bicornis*

offspring to the experimental temperatures for their entire development and transferred all bees concurrently to wintering temperatures, particularly the bees in the warm temperature treatments experienced a comparatively long pre-wintering period (due to accelerated development compared to the bees of the cooler temperature treatments) at relatively high temperatures. As a consequence, the mortality of the adult bees in the warm treatments was very high (chapter 2) – we were hardly able to obtain live bees for the investigation of brain structure in the first half of the overwintering phase in that year. We also examined the relative fat content of the bees that were used for brain preparations and found a strong and significant decrease in relative fat content with increasing temperature – under both constant and fluctuating conditions (N. Pichlmaier, S. Radmacher, E. Strohm, unpublished data). Since the prepupal weight of the bees in the fluctuating temperature treatments only slightly decreased with increasing rearing temperature (see above), the considerable loss of energy reserves in the warm temperature treatments (at least in the fluctuating one) might very likely be attributed to the long and warm pre-wintering phase. In 2009, we controlled the duration of the pre-wintering phase with regard to the duration of development by adjusting the onset of wintering temperatures individually for each temperature treatment (see chapter 5). Though we did not examine adult mortality of these bees systematically, we assume that it was generally low in all temperature treatments since we hardly found any dead adults inside their cocoons in the course of brain preparations (winter 2009) and the investigation of learning abilities (spring 2010). Moreover, the relative fat content of these bees was not (27.5°C) or only slightly (20-35°C) reduced in the warm temperature treatments (K. Weiß, K. Köllen, S. Radmacher, E. Strohm, unpublished data), indicating that the adjustment of the pre-wintering duration in the respective temperature treatments largely prevented exhaustive fat body depletion and, thus, elevated mortality.

However, in natural environment, the onset of wintering temperatures would not be adjusted (i.e. advanced) if warm summer temperatures might have accelerated bee development. In contrast, in addition to increasing overall temperatures and frequency of heat waves in summer that are likely to accelerate the development of insects (Hughes 2000), an extension of the growing season due to the current climate change is predicted and has been already reported (Bradshaw and Holzapfel 2001; Menzel et al. 2001, 2006; Schwartz et al. 2006; IPCC 2007; EEA 2008). Thus, spring (and bee development) starts earlier, but winter does not. As a consequence, the pre-wintering phase of the bees might be extended – with the above mentioned negative consequences for the bees. In *O. lignaria*, Sgolastra (2007) found

increased mortality in bees subjected to simulated late onset of winter. However, we found indications for an adaptive mechanism in *O. bicornis* that may allow the adjustment of pre-wintering duration through the adjustment of the timing of adult eclosion: The prepupal stage was extended when the bees experienced warm temperatures during larval development.

The prepupal stage seems to be a remarkable developmental stage in *Osmia* bees. Many hymenopteran species, including the most megachilid bee species, overwinter (and typically undergo a diapause) in the prepupal stage (Danks 1987; Westrich 1989; Bosch et al. 2001; Kemp et al. 2004). In contrast, *Osmia* bees overwinter in the adult stage which seems to be a derived trait within the Megachilidae (Bosch et al. 2001). Wintering in the adult stage allows early activity in spring since the bees do not have to complete their development when temperatures rise as it is the case for other Megachilidae, e.g. the summer-active *Megachile rotundata* that overwinters in the prepupal stage. A disadvantage of adult wintering might be that the adult stage seems to be generally more sensitive to warm temperatures than the prepupal stage: *M. rotundata* appears to be much more tolerant to warm or longer pre-wintering and wintering periods than *Osmia* (Richards et al. 1987; Kemp and Bosch 2001; Bosch and Kemp 2003, 2004; Pitts-Singer and James 2009; Bosch et al. 2010) and, unlike *Osmia* adults, *Osmia* prepupae can be kept at warm temperatures for many months and even years without dying (Bosch 1994; Bosch and Kemp 2000; Sgolastra 2007; Bosch et al. 2010). However, albeit *Osmia* bees do not use this obviously less temperature-sensitive prepupal stage for wintering, they probably undergo a summer diapause in this stage as indicated by lowered respiration rates (Kemp et al. 2004; Bosch et al. 2010). Weight loss in the prepupal stage is comparably low (compared to other developmental and life cycle phases; Bosch and Vicens 2002) though this stage may last 1-2 months (Bosch and Kemp 2000; Bosch and Vicens 2002; Bosch et al. 2000, 2010). The prepupal stage seems to be the stage with the largest plasticity in duration and the timing of adult eclosion depends largely on the duration of this stage (Sgolastra 2007; Bosch et al. 2010). Thus, this stage seems to be most appropriate for an active regulation of development time and the adjustment of adult eclosion to the onset of wintering temperatures. Johnston and Bennett (1996, prologue) stated that “species have been found to exhibit considerable plasticity in their responses to changing temperature” and that “this plasticity is evident both phenotypically for individual organisms and genotypically for populations and species during evolutionary adaptation to diverse thermal environments”. Bosch et al. (2000) reported indications for an evolutionary adaptive genotypic plasticity in the duration of the prepupal stage since, when reared under

the same conditions, *Osmia* bees of populations from lower latitudes (with longer and warmer growing seasons) remain longer in the prepupal stage than bees that originate from more northerly populations. In our studies, we found evidence for (adaptive) phenotypic plasticity in the duration of the prepupal stage in *O. bicornis* since the individual organism seems to be able to extend the prepupal stage in response to warm temperatures (and, thus, accelerated development) during larval development to prevent long pre-wintering periods with their negative consequences. Thus, this plasticity in the duration of the prepupal stage might help these important pollinators to cope with the increasing overall temperatures and frequency of heat waves in summer due to climate change.

However, though we found that, under fluctuating conditions, elevated temperatures during development (as might occur due to climate change) would probably have only small negative effects on prepupal weight and pre-wintering duration in *O. bicornis* (chapter 2 and 3), it cannot be concluded that climate change would probably not negatively affect this bee species with regard to body size, energy reserves, and overwintering survival. For reliable conclusions about possible effects of climate change on these traits, we would need to investigate the effects of several fluctuating temperature regimes in more detail (e.g. with temperature regimes that are more variable in minimum and maximum temperatures as well as in amplitude than in the studies presented here). In doing so, temperature effects on weight loss and duration of all distinct phases of development (including the pupal stage) and the other phases of the life cycle (including overwintering and the emergence period in spring) should be taken into account. Temperature effects on overwintering and emergence periods have already been investigated in megachilid bees (Tasei and Masure 1978; Richards et al. 1987; Bosch and Blas 1994; Rust 1995; Wilson and Abel 1996; Bosch et al. 2000; Kemp and Bosch 2000, 2001, 2005; Bosch and Vicens 2002; Bosch and Kemp 2003, 2004; Krunic and Stanisavljević 2006b; Yocum et al. 2006; Sheffield et al. 2008; Sgolastra et al. 2010). However, since most of these studies were conducted in the context of pollinator-management purposes, fluctuating temperature regimes have rarely been used. Since there are indications that fluctuating and constant temperatures may differ in their influences on overwintering megachilid bees (e.g. Yocum et al. 2006; Sheffield et al. 2008), the investigation of potential influences of several fluctuating temperature regimes on overwintering and the induction of emergence may be helpful for an estimation of potential consequences of climate change for *O. bicornis*.

7.2 Temperature effects on the brain and cognitive abilities

The brain of insects has shown considerable plasticity in response to conditions during (larval and pupal) development and adult maturation (reviewed in Meinertzhagen 2001; Groh and Meinertzhagen 2010). It is generally assumed that brain plasticity accompanies experience as well as learning and memory (Kolb and Wishaw 1998; Groh and Meinertzhagen 2010) which are essential for foraging performance in bees (Hammer and Menzel 1995; Menzel 2001; Dukas 2008). This suggests that brain plasticity is probably a generally advantageous trait for bees. However, regarding temperature influences during development, brain plasticity might be disadvantageous (see below).

In our studies, we focused on the mushroom bodies (MBs) in bee brains. These structures have a prominent function in insect learning, memory, and orientation (Erber et al. 1987; Menzel et al. 1994; Heisenberg 1998; Strausfeld et al. 1998, 2009; Zars 2000; Gronenberg 2001; Fahrbach 2006). Several studies revealed considerable plasticity in the MBs, particularly in the size of distinct neuropils (particularly the calyces) as a response to age, experience, behavioural transitions or social interactions in butterflies (KROUTOV et al. 2002), ants (Gronenberg et al. 1996), wasps (O'Donnell et al. 2004; Molina and O'Donnell 2007, 2008), honeybees (Withers et al. 1993; Durst et al. 1994; Fahrbach et al. 1997; Groh et al. 2006; Maleszka et al. 2009; Stieb et al. 2010), bumblebees (Riveros and Gronenberg 2010), sweat bees (Smith et al. 2010), and *Osmia lignaria* (Withers et al. 2008). Moreover, the synaptic organization in the MB calyces, i.e. size, density and number of microglomeruli (MG; distinct synaptic complexes), also exhibited considerable plasticity due to age, caste, experience, and learning in adult ants, honeybees, and cockroaches (Seid et al. 2005; Groh et al. 2006; Lent et al. 2007; Groh and Rössler 2008; Krofczik et al. 2008; Hourcade et al. 2010; Stieb et al. 2010). Usually, neuropil sizes as well as MG numbers increased with increasing experience and complexity of tasks or environment, with learning, and with age (but see Stieb et al. 2010). In contrast, temperature influences during development negatively affected neuropil size and MG number in MBs: In *Drosophila melanogaster*, ecologically-relevant heat stress during larval and pupal development resulted in a remarkable decrease in neuropil size in the MBs and negatively affected locomotor performance and associative odour learning in adult flies (Roberts et al. 2003; Wang et al. 2007). In honeybees, calyx neuropil sizes and MG number decreased when pupal rearing temperature deviated from the narrow optimal range of about 34-35°C which is usually maintained for pupal development

in the colony (Groh et al. 2004, 2006). Decreased MG numbers may be associated with poorer performance in dance behaviour and olfactory learning which were also detected in honeybees reared at non-optimal temperatures (Tautz et al. 2003; Jones et al. 2005; Groh and Rössler 2008; Groh and Meinertzhagen 2010).

In the solitary bee *O. bicornis*, we found no or rather small effects of developmental temperature on neuropil size, MG density and total MG number under constant as well as fluctuating conditions (chapter 4 and 5) – although particularly the temperature conditions during the pupal stage are likely to affect the physiological processes that underlie the complete remodelling of the central nervous system during metamorphosis (Bauer 1904; Truman 1990; Levine et al. 1995; Farris et al. 1999, 2004; Ganeshina et al. 2006) and, thus, the final brain structure of the adult either directly or indirectly via hormonal pathways (Weeks and Levine 1990; Fahrbach and Weeks 2002). Our results suggest that brain development of this solitary bee is buffered against temperature influences and, thus, exhibited a considerable degree of developmental stability. This is consistent with our hypothesis that solitary bees should be adapted and, thus, less susceptible to changing temperatures during development than honeybees. Social honeybees and solitary bees seem to use different strategies to achieve a high degree of stability for the development of the generally plastic brain: While honeybees keep ambient temperatures for their brood relatively constant (with high time and energy effort, see 1.2), solitary bee offspring seem to possess physiological mechanisms for buffering the effects of changes and fluctuations of environmental temperature to which they are naturally exposed.

However, we do not know the mechanisms that might be responsible for the buffering of brain development. We found indications for a compensation of temperature effects on neuropil size by a reciprocal effect on MG density, resulting in a more or less constant total MG number. However, this compensative mechanism was only observed in bees that were exposed to the experimental temperatures during their entire development (chapter 4) and not in bees that experienced the respective temperatures only during post-larval development (“PLD-*Osmia*”; chapter 5). Since the effects of pupal rearing temperature on neuropil size, MG density and particularly total MG number were still much smaller in PLD-*Osmia* than in honeybees, there must be further mechanisms that allow for a relatively stable brain development in a changing environment - and that might be investigated in future studies.

Consistent with our results regarding temperature influences on brain development, we did not detect any effect of rearing temperature on visual learning abilities in *O. bicornis* females (chapter 6). However, one might argue that the generally only moderate learning success that was exhibited by the bees during the experiment might have masked potential effects of developmental temperature on learning abilities. Preliminary tests had been more promising with regard to the general success in learning to associate a reward with yellow and in fast reversal learning. Based on these preliminary results, we used the respective training and test methods including differential conditioning (in which a bee was simultaneously confronted with both the rewarding colour and the non-rewarding colour during training) for the investigation of rearing temperature effects on the cognitive abilities of *O. bicornis*. However, studies on honeybees and bumblebees indicated that absolute conditioning, in which a subject is trained with a single colour rewarded with sugar water, yields generally faster learning (Dyer and Chittka 2004; Dyer and Neumeyer 2005; Giurfa 2004; Avarguès-Weber et al. 2011). Thus, the overall learning success might probably be improved using absolute conditioning instead of differential conditioning in future studies. Moreover, rearing temperature effects on learning and memory in honeybees were demonstrated with regard to olfactory learning (Tautz et al 2003; Jones et al. 2005). Since different neuropils in the bee brain are involved in processing olfactory and visual input (Mobbs 1982, 1985) and may be affected differentially by temperature or other influences (Groh et al. 2004; Groh and Meinertzhagen 2010), the investigation of temperature influences on olfactory learning in *O. bicornis* could probably serve to complete the knowledge about temperature influences on the brain and the cognitive abilities of solitary bees.

Though the methods for the investigation of learning abilities in *O. bicornis* might be improved and supplemented with regard to the investigation of temperature influences on cognitive abilities of solitary bees, we have shown that this solitary bee is generally able to overcome an innate colour preference by learning and to pass a task of fast reversal learning successfully. Thus, the solitary *O. bicornis* has exhibited considerable cognitive capacities. The morphologically complex and elaborated mushroom bodies that were found in social Hymenoptera have often been related to sociality in insects (von Alten 1910, Howse 1975, Gronenberg and Riveros 2009, Farris and Schulmeister 2011) and due to the complex demands of a social life (recognition of and communication with colony members) it has been stated that social species have more developed cognitive abilities than corresponding solitary species (social complexity theory; Dukas and Real 1991; Kamil 2004). In contrast

to this theory, there is evidence that the evolution of elaborated mushroom bodies and, thus, remarkable cognitive abilities, may be associated with other traits than sociality (Farris and Schulmeister 2011). Regarding bees, both social and solitary species are central place foragers that require spatial cognition abilities that enable them to find food in an unpredictable environment and to repeatedly return to their own nest (Menzel 2001; Amaya-Márquez and Wells 2008). Thus, there is no reason to believe that social bees are in any way special in their cognitive capacities – only because most studies concerning cognitive capabilities of insects were conducted with social bees (Menzel et al. 2001). Our studies on *O. bicornis* as well as other studies on the brain and cognitive abilities of solitary bees (von Alten 1910; Menzel et al. 1988; Steinmann and Menzel 1990; Perez and Waddington 1996; Campan and Lehrer 2002; Amaya-Márquez and Wells 2008; Amaya-Márquez et al. 2008; Withers et al. 2008; Vorel 2010) indicated that solitary bees also possess complex elaborated mushroom bodies and considerable learning capacities, probably comparable to honeybees. It might be more difficult for us to test for learning capacities in solitary bees than in social bees (e.g. due to the failure of PER-conditioning in solitary bees; Vorel and Pitts-Singer 2010; chapter 6) – however, that does not necessarily mean that the cognitive abilities of solitary bees are inferior to that of social species.

7.3 Conclusive remarks

Our studies on *O. bicornis* revealed considerable plasticity in body size and development time in response to different temperature treatments during development. In contrast, brain development seems to be largely buffered against temperature influences and, thus, rather stable. Furthermore, we detected no effect of developmental temperature on cognitive capacities. Our results suggest that *O. bicornis* might use temperature during larval development as a cue for temperature conditions in their environment that allows the adjustment of several physiological processes in subsequent developmental stages to the prevailing conditions. The developmental plasticity in the duration of the prepupal stage and the developmental stability regarding important neuropils in the brain may be considered to be adaptive since the offspring of solitary bees are largely exposed to changing and in part unpredictable ambient temperatures during development.

However, though our results might indicate that *O. bicornis* might be well-equipped to cope with changing and unpredictable temperatures during development, we are careful in drawing any conclusions about potential consequences of climate change on this abundant solitary bee species since temperature influences during the whole life cycle should be taken into account for such purposes. Moreover, we investigated temperature influences during development only on a selected sample of important life history traits – temperature during development (and, thus, climate change) might affect other fitness-relevant traits as well. For example, larval rearing temperature influenced amount and composition of the marking pheromone of the territorial males in the digger wasp *Philanthus triangulum* (Roeser-Mueller et al. 2010). Moreover, increasing temperatures can negatively affect immune function in insects (Karl et al. 2011). In *O. bicornis*, we have already found that the amount and composition of hydrocarbons on the cuticula of adult bees are affected by developmental temperature (N. Pichlmaier, K. Weiß, K. Köllen, S. Radmacher, E. Strohm, unpublished data) – and cuticular hydrocarbons (on males) play a role in mate choice in this species (Conrad et al. 2010). Thus, the investigation of potential (rearing) temperature effects on reproductive success of males (i.e. number of mates) and females (i.e. number and quality of brood cells/ surviving offspring) or e.g. on immune function and/or induction of emergence in spring (and, thus, synchronization with bloom of main host plants) might give further insights in the temperature dependence of *O. bicornis* that might help to estimate potential consequences of climate change for this important European pollinator.

CHAPTER 8

SUMMARY

Temperature is one of the most important environmental factors for ectotherms and the temperature experienced during development affects many important life history traits in insects, e.g. body size, development time, and fecundity. Since ambient temperatures are highly variable, particularly in temperate regions, insects may experience considerable temperature variations during development. The eusocial honeybees maintain a more or less constant temperature for their brood (33-36°C, mostly around 34-35°C) via active thermoregulation. When temperatures during development were experimentally manipulated, warmer or cooler temperatures had clear negative effects like elevated mortality and malformations. Even small deviations from the optimal temperature range of 34-35°C affected the synaptic organization in the brain, behavioural performance, and learning and memory in adult honeybees.

In contrast to honeybees, most bee species are solitary. Since there is no active regulation of brood temperature in solitary bees, their offspring are largely exposed to the fluctuating ambient temperatures (at least in cavity-nesting species). If solitary bees showed the same negative effects of deviations from the optimal temperatures during development as honeybees, they would suffer huge fitness losses. Thus, solitary bees should have adapted to fluctuating and changing temperatures during development. However, little seems to be known about temperature requirements of solitary bees under fluctuating conditions or any adaptive mechanisms that might help these bees to cope with variable and often unpredictable temperatures during development so far.

In this thesis, we investigated potential influences of temperature during development on body size, development time, synaptic organization in the brain, and cognitive abilities of the solitary Red mason bee, *Osmia bicornis* (Hymenoptera, Megachilidae). Since the responses to developmental temperature might differ between constant and fluctuating temperatures and since solitary bee offspring should be adapted to fluctuating rather than constant temperature conditions, we used three fluctuating (10-25, 15-30, and 20-35°C) as well as three constant temperature treatments (17.5, 22.5, and 27.5°C) plus one control group as a reference for

natural temperature conditions. Moreover, since the temperature experienced during larval development might influence the thermal responses of subsequent developmental stages, we varied the duration of exposure to the different temperature treatments: In one year, *O. bicornis* offspring were exposed to the experimental temperatures during their entire development. In the following year, the respective temperature treatments were only applied during development inside the cocoon (i.e. the prepupal and the pupal stage).

In general, body size (i.e. prepupal weight) decreased with increasing temperature during larval development, but not as strong under fluctuating conditions. Bees attained higher prepupal weights under fluctuating conditions than in the correspondent constant temperature treatment. Increasing and fluctuating (vs. constant) temperatures accelerated development in almost all stages and temperature regimes. However, the prepupal stage was prolonged in the warm temperature treatments, but only in bees that had experienced these temperatures during entire development. Thus, the extension of the prepupal phase might be a mechanism to adjust adult eclosion to the onset of wintering temperatures when larval development was accelerated due to hot summers. This mechanism might help to avoid long pre-wintering periods with their negative effects (e.g. elevated mortality) on the adult bees inside their cocoons.

Regarding potential temperature effects on the brain of *O. bicornis*, we focused on the synaptic organization in the calyces of the mushroom bodies (MBs). The MBs are prominent neuropils in the insect brain that play an important role for cognitive skills (learning, memory, and orientation) which are probably crucial for the foraging performance in bees. We analysed temperature effects on neuropil size as well as density and number of distinct synaptic complexes, the microglomeruli (MG), in the calyces and compared our results with data on honeybees. Temperature affected neuropil size, regardless whether the experimental temperatures were applied during entire development or only during post-larval development. However, this effect was largely compensated by a reciprocal effect on MG density – but only in bees that had experienced the experimental temperatures during entire development. As a result, overall MG numbers were hardly affected by developmental temperature in these bees. However, though we did not detect such a compensatory effect on MG density in bees that were exposed to the experimental temperatures only during post-larval development, the temperature effects on the brain, particularly on overall MG numbers, were considerably and significantly smaller in all experimental groups of *O. bicornis* than in honeybees.

In this thesis we developed a visual learning paradigm for *O. bicornis* to test whether temperature during post-larval development might influence the learning abilities of the bees. We found evidence for an innate preference for blue, but the bees were generally able to overcome this innate preference by learning to associate a reward with yellow. We did not detect an effect of developmental temperature on the abilities for visual learning and fast reversal learning. This is consistent with the above mentioned result that, in *O. bicornis*, brain structure is much less affected by developmental temperature than in honeybees. Our results support the hypothesis that, in contrast to honeybees, solitary bees are adapted and, thus, less susceptible to fluctuating temperatures during development.

Our studies on *O. bicornis* revealed considerable plasticity in body size and development time in response to different temperature treatments during development. In contrast, brain development seems to be largely buffered against temperature influences and, thus, rather stable. Our results suggest that *O. bicornis* might use temperature during larval development as a cue for temperature conditions in their environment that allows the adjustment of several physiological processes in subsequent developmental stages to the prevailing conditions. Developmental plasticity in the duration of the prepupal stage and developmental stability regarding important neuropils in the brain may be considered to be adaptive since the offspring of solitary bees are largely exposed to variable and in part unpredictable ambient temperatures during development. The detected differences in thermal responses to fluctuating vs. constant temperatures illustrated the importance of fluctuating temperatures in studies about ecologically-relevant temperature influences on insects.

CHAPTER 9

ZUSAMMENFASSUNG

Temperatur ist einer der bedeutendsten Umweltfaktoren für Ektotherme und die Temperatur während der Entwicklung beeinflusst viele wichtige Lebenslaufparameter („life history traits“) von Insekten, z.B. Körpergröße, Entwicklungsdauer und Fekundität. Da die Umgebungstemperaturen besonders in den gemäßigten Breiten sehr variabel sind, können Insekten erhebliche Temperaturschwankungen während ihrer Entwicklung erfahren. Die eusozialen Honigbienen halten mittels aktiver Thermoregulation die Temperatur für ihre Brut mehr oder weniger konstant (33-36°C, meist bei 34-35°C). Wurde die Temperatur während der Entwicklung experimentell manipuliert, so hatten wärmere und kältere Temperaturen deutlich negative Auswirkungen wie erhöhte Mortalität und Missbildungen. Sogar nur geringe Abweichungen vom optimalen Temperaturbereich von 34-35°C wirkten sich auf synaptische Strukturen im Gehirn, das Verhalten sowie Lernen und Gedächtnis von adulten Honigbienen aus.

Im Gegensatz zu Honigbienen sind die meisten Bienenarten solitär. Da bei solitären Bienen keine aktive Regulation der Temperatur für die Brut betrieben wird, ist ihr Nachwuchs weitgehend den schwankenden Umgebungstemperaturen ausgesetzt (zumindest bei oberirdisch nistenden Arten). Würden bei solitären Bienen die gleichen negativen Effekte durch Abweichungen von den optimalen Entwicklungstemperaturen auftreten wie bei Honigbienen, so würden sie hohe Fitness-Einbußen erleiden. Deshalb sollten sich solitäre Bienen an fluktuierende und unbeständige Temperaturen während der Entwicklung angepasst haben. Bisher scheint jedoch wenig über die Temperaturansprüche von solitären Bienen unter fluktuierenden Bedingungen oder mögliche adaptive Mechanismen, die es diesen Bienen erlauben mit variablen und wechselhaften Entwicklungstemperaturen zurechtzukommen, bekannt zu sein.

In dieser Arbeit untersuchten wir mögliche Einflüsse der Entwicklungstemperatur auf Körpergröße, Entwicklungsdauer, synaptische Strukturen im Gehirn und kognitive Fähigkeiten bei der solitären Roten Mauerbiene, *Osmia bicornis* (Hymenoptera, Megachilidae). Da sich

die Reaktionen auf die Entwicklungstemperatur zwischen konstanten und fluktuierenden Temperaturen unterscheiden könnten und weil der Nachwuchs von solitären Bienen eher an fluktuierende als an konstante Temperaturbedingungen angepasst sein sollte, verwendeten wir drei fluktuierende (10-25, 15-30 und 20-35°C) und drei konstante (17,5, 22,5 und 27,5°C) Temperaturbehandlungen sowie eine Kontrollgruppe als Referenz für natürliche Bedingungen. Da die während der Larvalentwicklung erfahrene Temperatur die Reaktionen auf die Temperatur in nachfolgenden Entwicklungsstadien beeinflussen könnte, variierten wir die Dauer der Temperaturbehandlungen: In einem Jahr wurde *O. bicornis*-Nachwuchs während der gesamten Entwicklung den Versuchstemperaturen ausgesetzt, im folgenden Jahr nur während der Entwicklung im Kokon (d.h. dem Präpuppen- und Puppenstadium).

Die Körpergröße (Präpuppengewicht) nahm grundsätzlich mit steigender Temperatur während der Larvalentwicklung ab, allerdings unter fluktuierenden Bedingungen nicht so stark. Die Bienen erreichten bei fluktuierenden Bedingungen ein höheres Präpuppengewicht als in den entsprechenden konstanten Temperaturbehandlungen. Steigende und fluktuierende (vs. konstante) Temperaturen beschleunigten die Entwicklung in fast allen Stadien und Temperaturbereichen. Das Präpuppenstadium allerdings war in den warmen Temperaturbehandlungen verlängert, jedoch nur bei den Bienen, die diese Temperaturen während ihrer gesamten Entwicklung erfahren hatten. Somit könnte die Verlängerung der Präpuppenphase ein Mechanismus zur Anpassung des Zeitpunkts des Erreichens des Adultstadiums an den Beginn des Winters sein, wenn die Larvalentwicklung aufgrund heißer Sommertemperaturen beschleunigt wurde. Dieser Mechanismus könnte dazu beitragen, lange Vorwinterphasen mit ihren negativen Auswirkungen (z.B. erhöhte Mortalität) auf die adulten Bienen in ihren Kokons zu vermeiden.

Was mögliche Temperatureinflüsse auf das Gehirn von *O. bicornis* anbelangt, so konzentrierten wir uns auf synaptische Strukturen innerhalb der Calyces der Pilzkörper. Die Pilzkörper sind bekannte Neuropile im Insektengehirn. Sie spielen eine bedeutende Rolle für kognitive Fähigkeiten (Lernen, Gedächtnis und Orientierung), die wahrscheinlich sehr wichtig für den Fouragier-Erfolg von Bienen sind. Wir analysierten Temperatureffekte auf Neuropilgröße sowie Dichte und Anzahl bestimmter synaptischer Komplexe, den Mikroglomeruli (MG), in den Calyces und verglichen unsere Ergebnisse mit entsprechenden Daten für Honigbienen. Die Temperatur beeinflusste die Neuropilgröße, unabhängig davon, ob die Versuchstemperaturen während der gesamten oder nur während der post-larvalen

Entwicklung eingesetzt wurden. Dieser Effekt wurde jedoch weitgehend durch einen reziproken Effekt auf die MG-Dichte kompensiert, sodass schließlich die Gesamtanzahl der MG kaum durch die Entwicklungstemperatur beeinflusst wurde – jedoch nur bei den Bienen, die die Versuchstemperaturen während ihre gesamten Entwicklung erfahren hatten. Auch wenn wir bei den Bienen, die den Versuchstemperaturen nur während ihrer post-larvalen Entwicklung ausgesetzt waren, keinen reziproken Effekt auf die MG-Dichte finden konnten, so waren generell die Temperatureffekte auf das Gehirn – besonders auf die MG-Anzahl – in allen *O. bicornis*-Versuchsgruppen deutlich und signifikant kleiner als bei Honigbienen.

In dieser Arbeit entwickelten wir ein Lernparadigma für visuelles Lernen in *O. bicornis*, um zu prüfen, ob sich die Temperatur während der post-larvalen Entwicklung auf die Lernfähigkeiten der Bienen auswirken könnte. Wir fanden Anzeichen für eine angeborene Präferenz für Blau, die Bienen waren jedoch grundsätzlich in der Lage, diese Präferenz zu überwinden, indem sie lernten, eine Belohnung mit Gelb zu assoziieren. Wir fanden keinen Effekt der Entwicklungstemperatur auf die Fähigkeiten für visuelles Lernen und schnelles Umlernen. Dies steht im Einklang mit dem oben erwähnten Ergebnis, dass die Feinstruktur im Gehirn bei *O. bicornis* wesentlich schwächer durch die Entwicklungstemperatur beeinflusst wird als bei Honigbienen. Unsere Ergebnisse stützen die Hypothese, dass solitäre Bienen – im Gegensatz zu Honigbienen – an schwankende Temperaturen während der Entwicklung angepasst und somit diesbezüglich weniger beeinflussbar sind.

Unsere Untersuchungen zeigten eine beachtliche Plastizität der Körpergröße und der Entwicklungsdauer als Reaktion auf verschiedene Temperaturbehandlungen während der Entwicklung. Im Gegensatz dazu scheint die Gehirnentwicklung weitgehend gegen Temperatureinflüsse gepuffert und somit ziemlich stabil zu sein. Unsere Ergebnisse deuten darauf hin, dass *O. bicornis* die Temperatur während der Larvalentwicklung als Signal für die Temperaturbedingungen in der Umgebung nutzen könnte, das die Anpassung einiger physiologischer Prozesse der folgenden Entwicklungsstadien an die vorherrschenden Bedingungen ermöglicht. Die Plastizität bezüglich der Dauer der Präpuppenphase und die Stabilität in der Entwicklung wichtiger Neuropile im Gehirn können als adaptiv angesehen werden, da der Nachwuchs solitärer Bienen während der Entwicklung natürlicherweise weitgehend den schwankenden und teilweise unvorhersagbaren Umgebungstemperaturen ausgesetzt ist. Die gefundenen Unterschiede in den Auswirkungen von fluktuierenden und konstanten Temperaturen unterstreichen die Bedeutung von fluktuierenden Temperaturen in Studien über ökologisch relevante Temperatureinflüsse bei Insekten.

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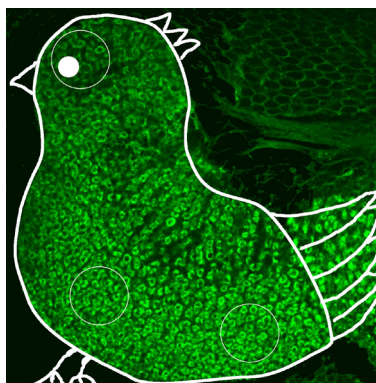
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